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Effects of Selected Waterborne Particulates on Cellular and Molecular Parameters

Ohio State Univ., Columbus

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Health Effects Research Lab.
Research Triangle Park, NC

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EFFECTS OF SELECTED WATERBORNE PARTICULATES ON CELLULAR AND MOLECULAR PARAMETERS

by

R.W. Hart, R. Fertel, H.A.I. Newman, J.R. Blakeslee
Chemical Biomedical Environmental Research Group
The Ohio State University
Columbus, Ohio 43210

Grant No. R804201

Project Officer

James R. Millette
Toxicology and Microbiology Division
Health Effects Research Laboratory
Cincinnati, Ohio 45268

HEALTH EFFECTS RESEARCH LABORATORY
OFFICE OF RESEARCH AND DEVELOPMENT
U.S. ENVIRONMENTAL PROTECTION AGENCY
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16. ABSTRACT

Embryonic cultures were used to compare the effects of six particulate samples, some filtered directly from drinking water on a cellular, biochemical and molecular basis. Cytotoxicity was exhibited in human cells for all agents tested at the highest concentration used (10 mg/ml). All particulates from city drinking waters were less toxic than commercial chrysotile asbestos. The particulate samples had varying effectiveness in reducing cell membrane surface complex glycolipids and high molecular mass glycoproteins. Three particulate samples were collected from drinking waters from Duluth, MN, San Francisco, CA and Seattle, WA. The other three samples included less than 2 micrometer size fraction of taconite tailings, attapulgite clay, and particulates collected after an asbestos-cement pipe was tapped.

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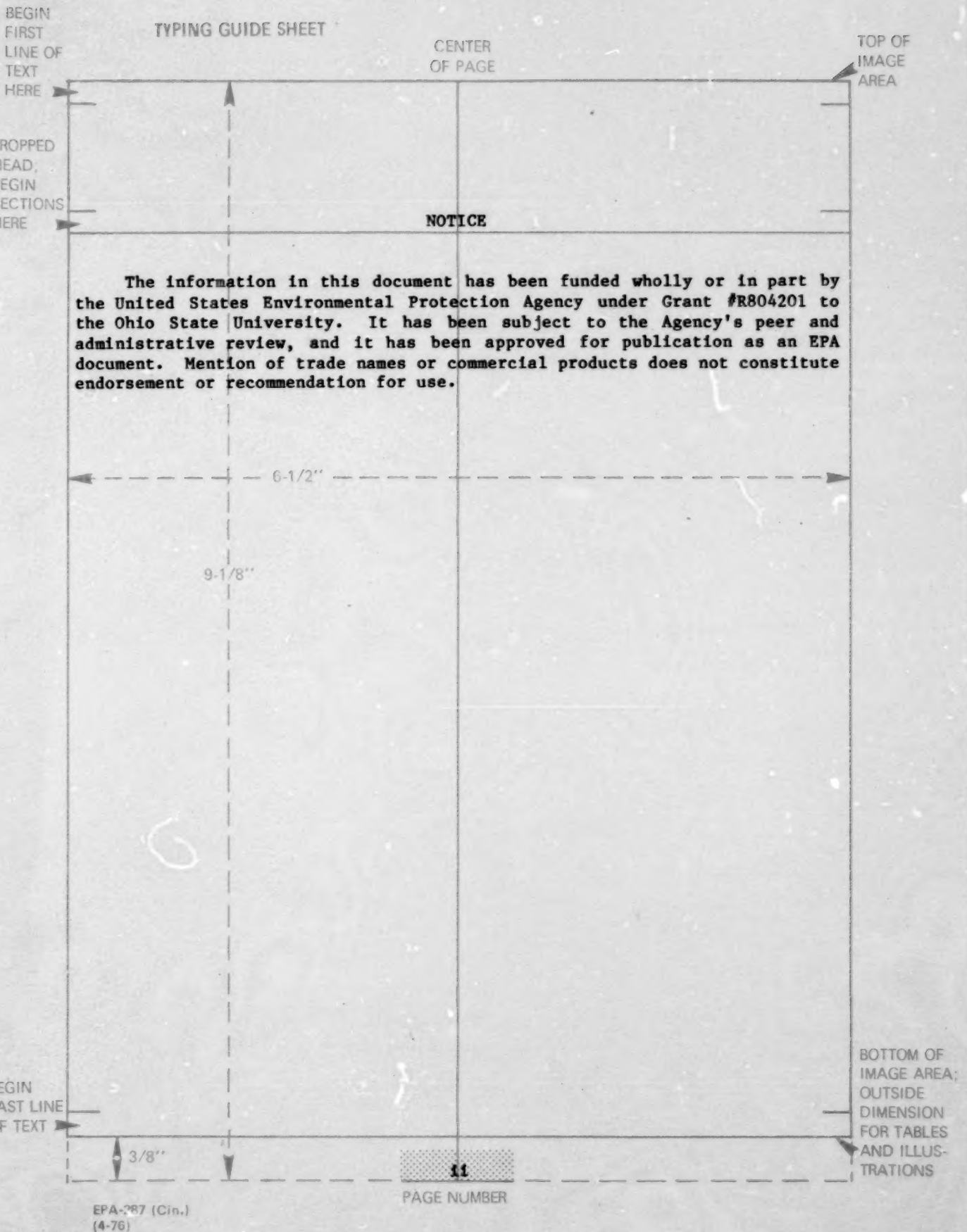
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FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The complexities of environmental problems originate in the deep interdependent relationships between the various physical and biological segments of man's natural and social world. Solutions to these environmental problems require an integrated program of research and development using input from a number of disciplines. The Health Effects Research Laboratory, Research Triangle Park, NC and Cincinnati, OH conducts a coordinated environmental health research program in toxicology, epidemiology and clinical studies using human volunteer subjects. Wide ranges of pollutants known or suspected to cause health problems are studied. The research focuses on air pollutants, water pollutants, toxic substances, hazardous wastes, pesticides and non-ionizing radiation. The laboratory participates in the development and revision of air and water quality criteria and health assessment documents on pollutants for which regulatory actions are being considered. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of environmental regulatory decisions involving the protection of the health and welfare of all U.S. inhabitants.

The report that follows describes effects of various particulate samples, some directly from drinking water supplies, on biological endpoints in cultured mammalian cells. An understanding of the effects of particles on the individual cell is important in determining the potential effects on health of particulates in drinking water.

F. Gordon Hueter
Director
Health Effects Research Laboratory

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PREFACE

Mineral fibers and particulates represent some of the most ubiquitous environmental contaminants known. They are found in air, soil, and water and thus reach man by a number of vectors. Since many of these materials are relatively inert they do not break down, but rather recirculate in the environment. However, the most important aspect of mineral fibers and particulates is that modern day science has yet to determine their mode of action or to develop a method other than epidemiology to screen for their potential human health effects.

Previous studies performed independently by our group and other laboratories have suggested that asbestos induces various biochemical alterations in cells in culture, and further that different types of asbestos modify these same parameters to differing extents. Since the extent of the effect on certain of these parameters for various forms of asbestos appears to correlate to some degree with the carcinogenic potential of these agents, as determined by epidemiological studies, it was deemed reasonable that a double-blind study be performed to further examine this correlation with unknown samples, which may be found or isolated from drinking water.

In regards to this mission, the CBERG program of The Ohio State University has contributed, via its multidisciplinary approach, studies on:

- cytotoxicity
- modification of virally-directed cellular transformation
- the alteration of cell membrane composition
- modification of cyclic nucleotide ratios.

The report that follows describes effects of varying dosage and, in many cases, time of application of these unknowns on the above biological endpoints in cultured mammalian cells.

Since the exact nature of the samples was unknown, a prediction as to the screening potential of any of these measurements is yet to be elucidated. It can be stated, however, that all substances tested had at differing dose levels some degree of cytoxicity in human cells. Further, that the substances had, to a varying degree, an effect on the ratio of cyclic AMP/cyclic GMP, but in differing directions. The same was true regarding their effect on modification of membrane structure and virally-directed cellular transformation. These studies taken in combination with our previous findings and those of other laboratories

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suggest that mineral fibers and particulates may have two effects: a) cytotoxic and b) promotional, with their primary role in carcinogenesis being the latter. Their role in cytotoxicity, however, may be an indicator of their promotional activity.

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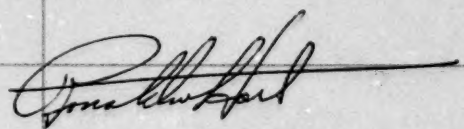

Ronald W. Hart, Ph.D.
The CBERG Group

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ABSTRACT

The purpose of this grant was to examine six unknown samples of mineral fibers and/or particulates isolated from drinking water supplies in order to determine if any of the four cellular and biochemical parameters measured would permit a prediction as to their potential human health risk.

The test systems employed were a normal human fibroblast cell strain established at Columbus and primary Syrian hamster embryonic cell cultures. The cellular and biochemical parameters examined were: 1) cytotoxicity, 2) virally-directed cellular transformation, 3) cell membrane composition, and 4) cyclic nucleotide concentrations.

Results of these studies were expressed as percentage of controls for each of the variables measured. Cytotoxicity was exhibited in human cells for all agents tested at the highest concentration used (10 mg/ml). The variation in colony morphology was examined, its dose-response determined and shown to be consistent for each type of colony examined relative to the overall dose-response curve exhibited by the culture as a whole. The width of the shoulder and particularly the slope of the exponential part of the curve varied as a function of the substance examined.

Whereas all six known asbestos samples previously examined increased the frequency of Snyder-Theilen Feline Sarcoma Virus transformation of human cells in vitro to one degree or another, our unknown samples did not uniformly do so. Samples 1,2,4,5, and 6 significantly inhibited viral transformation when cells were treated 2 hours prior to infection whereas substance 3 enhanced transformation at this same time point. Substance 4 at a concentration of 0.1 µg/ml enhanced tranformation when applied 24 hours prior to infection. All substances, with the exception of 6, affected virus-directed transformation in a dose-dependent manner.

Relative to the effect of unknown samples on the cyclic nucleotide response of cells with increasing doses of asbestos, no predictable dose-response relationship was noted; however, there were striking differences in the tissue response of human cells to the different samples examined. These responses could be divided into two (2) groups with samples 1,2 and 3 having relatively minor effects and samples 4,5 and 6 causing a consistent decrease in the cyclic nucleotide ratio at all concentrations tested. The most striking effects were seen with sample 4. The only sample studied which exhibited an effect similar to those observed previously with known samples of asbestos was sample 3 which exhibited the biphasic curve observed previously. Interestingly, the

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striking decrease in ratio observed with certain of these samples was very similar to that obtained with chemical carcinogens.

Cell membrane monosialogangliosides (GM_1, GM_2) are an index of a simpler cell surface glycolipid pattern which in previous studies with known samples appeared to change in a direction which was approximately equivalent to the carcinogenic potential of the samples examined.

In the present studies, samples 1 and 2 caused little change in the proportions of surface gangliosides whereas samples 3,4, and 5 produced losses in surface GT_1 with the greatest effect being manifested on the surfaces of cells treated with samples 4 and 5. The chrysotile-incubated cells included in the sample 6 study reacted anomalously to the asbestos making the interpretation of the sample 6 changes difficult. When sample 6 treated cells were compared to the control for samples 3-5, there was a loss in GD_{1A} and a corresponding increase in GM_1. GL-4 in the neutral glycolipids increased with samples 1 and 2 treatment and decreased with samples 3 and 4 cell incubations. Consistent with its actions on gangliosides, among the test samples, sample 4 caused the greatest reductions in GL-4. Again, sample 6 effects were anomalous due to the differences in the control distributions of glycolipids being so different from the sample 3-5 control.

Very high molecular weight proteins increased with cell treatment with samples 1 and 2. The control with samples 3,4 and 5 predominantly had a high concentration of a high molecular mass glycoprotein on the cell surfaces. Samples 3-6 allowed the detection of some of this glycoprotein, but it was in a lower concentration than that found in the controls. The loss of such high molecular mass proteins has been correlated with cells undergoing transformation.

This report was submitted in fulfillment of Grant No. R-804201-02 by the Chemical Biomedical Environmental Research Group under the sponsorship of the U.S. Environmental Protection Agency covering the period July 1, 1978 to April 15, 1979 and completed June 15, 1979.

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ABBREVIATIONS AND SYMBOLS

AMP	-- adenosine 3',5' - cyclic monophosphate
DEAE	-- diethyl amino ethyl
DMSO	-- dimethylsulfoxide
DNA	-- deoxyribonucleic acid
EDTA	-- dethylenedinitrilo-tetraacetic acid
EMEM	-- Earle's minimal essential medium
FBS	-- fetal bovine serum
FFU	-- focus forming units
GD_{1A}	-- disialoganglioside
GL-4	-- globoside
GM_1	-- monosialoganglioside
GM_2	-- monosialoganglioside
GMP	-- guanosine 3',5'-cyclic monophosphate
GT_1	-- trisialoganglioside
3H-TdR	-- tritiated thymine
L15	-- Leibovitz media
PBS	-- phosphate buffered saline
SDS	-- sodium dodecyl sulfate
SHE	-- Syrian hamster embryo
STFe SV	-- Snyder-Theilen Feline Sarcoma virus
TCA	-- trichloroacetic acid
TLC	-- thin-layer chromatography

CO_2	-- carbon dioxide
mCi	-- milli Curie
mg	-- milligram
ml	-- milliliter
mM	-- milli Molar
ng	-- nanogram
pH	-- -log hydrogen ion concentration
µg	-- microgram

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SECTION 1

INTRODUCTION

OBJECTIVES

The primary objective of this program was to perform a carefully controlled blind experiment in which a series of unknowns and coded samples were provided for examination to the CBERG program. Specifically, the purpose of this project was to evaluate the effect of these on:

1) virally-directed cellular transformation
2) cyclic nucleotide ratio
3) cell survival
4) membrane composition

Each of these parameters was previously found to be altered by known samples of asbestos (1); it, therefore, was deemed of interest to determine, in a blind study, if any of these biological parameters could be used to screen for the presence and human health effect of asbestos in water supplies.

PROJECT OFFICER'S NOTE

Samples of particulates, some extracted directly from drinking waters were sent to the grantee referenced only by a code number. The testing on the various cell parameters was then done "blind."

Samples 1,2, and 3 were particulates collected by filtration from drinking waters from Duluth, Seattle, and San Francisco, respectively. The Duluth sample was taken prior to the installation of the filtration plant at Duluth. Chrysotile fibers have been identified in the particulate samples from San Francisco and Seattle; amphibole fibers were identified among the particulates from the Duluth sample. Sample 4 was a sample of the less than 2 micrometer size fraction of taconite tailings which had been prepared by a sedimentation separation procedure. Amphibole fibers were identified among the particulates in sample 4. Sample 5 was a sample of attapulgite clay, a non-asbestos mineral, which contained short fibers in the same range as some chrysotile asbestos. Attapulgite fibers have been identified in some water supplies in Georgia and Florida. Sample 6 consisted of particulates collected from water flowing through asbestos cement pipe after the pipe was tapped. Sample 6 contained chrysotile, some crocidolite and cement pieces.

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SECTION 2

CONCLUSIONS

RELEVANCE OF PRESENT STUDIES TO CARCINOGENIC EFFECTS OF ASBESTOS

1. All unknown substances tested in the human cell-virus transformation assay system affected the frequency of virus transformation to varying degrees.

2. Relative to the ratio of cyclic nucleotides, only sample 3 enhanced the ratio while samples 4,5, and 6 resulted in a general decrease and samples 1 and 2 yielded no effect. Thus, the majority of these samples behaved in a manner similar to what would be expected if the cells had been treated with a chemical carcinogen.

3. The degree of cytotoxicity varied tremendously between samples with the concentration of substance needed to induce an LD_{50} being Canadian chrysotile >1 >6 >2 >5 >4.

4. Relative to glycolipids and glycoproteins, samples 1 and 2 promoted increases in GM_1 and GT_1 and GL-4 with concommitant increases in the high molecular mass band of surface glycoproteins whereas samples 3-6 had produced consistent decreases in G_{T1} and relative decreases in GL-4 with concommitant decreases in the high molecular mass band of surface glycoproteins.

Thus, it is apparent that these samples induced differing effects relative to the biological endpoints examined. It is still to be determined which of these endpoints is the most useful in predicting potential health hazards of waterborne particulates.

RELATIVE HAZARDS OF ONE UNKNOWN SAMPLE VERSUS ANOTHER

Of the unknown samples tested, samples 1,6, and 2 appeared in that order to be the most toxic. Relative to the effect of these substances on the cyclic nucleotide ratio, samples 1,2, and 3 had little effect whereas samples 4,5, and 6 produced a decrease in the ratio similar to that induced by chemical carcinogens. The effect of these samples on viral-directed transformation was inhibitory unlike the asbestos fibers previously studied (1) which were stimulatory with the exception of substance 3 which was stimulatory when cells were treated 2 hours before infection.

The order of effectiveness in reduction of surface complex gylcolipids was 5 >6 >3 >4 >2 = 1. The order of effectiveness in reduction of the high molecular mass glycoproteins was 5 >4 >3 >6 >2 >1.

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SECTION 3

NARRATIVE

METHODS

General

Cell Culture--

Cultures are maintained in conventional CO_2 incubators at 37°C and routinely tested for pleuro-pneumonia-like organisms. Cells were grown in Earle's Minimal Essential Medium (EMEM) supplemented with 1 mM sodium pyruvate, 2 mM glutamine, 1 percent non-essential amino acids and 10 percent fetal bovine serum. Confluent monolayers were dispersed with trypsin plus methyl cellulose. The determination of the generation time and various cell cycle parameters was made by standard autoradiographic procedures (2-5).

Cytotoxicity

Human Fibroblast Cell Cultures--

The general procedure we use for determining cytotoxicity via colony formation has been published previously (6). For these studies preconfluent monolayers are dispersed by trypsin plus methyl cellulose and triturated to assure single cell suspension. Two hundred and fifty, five hundred, or one thousand cells were plated into 100 mm diameter wells in EMEM supplemented with 10 percent fetal bovine serum, 1 mM sodium pyruvate, 2 mM glutamine, 1X nonessential amino acids (growth medium) and incubated for eight hours. Following attachment the unknown sample was added to varying concentrations to the cells for 24 hours. At the end of the incubation period, cells were washed and refed with growth medium. Cell cultures were incubated for 4 weeks, stained with crystal violet and clones enumerated as a function of number and size. Absolute and relative plating efficiencies were determined and survival curves calculated from the data.

Modification of Virally-Directed Transformation

Virus--

Briefly, 10 percent cell-free tumor homogenates from ST FeSV infected cats were prepared and stored in L-15 medium + 15 percent fetal bovine serum (FBS) at -70°C in 1 ml aliquots.

Infectivity Assay--

Cells were trypsinized and seeded onto either 16 mm (4×10^4 cells)

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or 35 mm (1 x 10^5 cells) diameter wells (Costar, Cambridge, MA) in 1.0 ml EMEM medium for the former and four ml for the latter and incubated 18 hours prior to treatment. Cells pretreated with particulates prior to virus infection were incubated with designated concentrations for two, six, or 24 hours, washed and treated with 0.2 ml (11 mm wells) or 1.0 ml (35 mm wells) of DEAE dextran (40 µg/ml) in serum-free EMEM. After twenty minutes, the cells were rinsed with EMEM + 5 percent FBS, infected at 0.05 ml per 16 mm wells or 0.2 ml per 35 mm wells with each of four twofold virus dilutions and allowed to absorb for two hours. Four wells were used per dilution of virus. The plates were rocked at 10 to 15 minute intervals to maintain an even distribution of inoculum and, after adsorption, the inoculum was removed and replaced with two ml or four ml of growth medium. Cells posttreated with particulates after virus infection were incubated with designated concentrations of chemicals two, six, or 24 hours after virus adsorption. The medium was removed from infected cells and the cells treated with medium containing only particulates for 24 hours, washed and refed with growth medium. Cells were refed with fresh growth medium only on the sixth day after infection and subsequently fixed with buffered formalin and stained with Giemsa three to four days later. Foci appear as discrete areas consisting of round, hyperrefractile, enlarged fibroblast cells. These foci were counted at 25 to 40 times with a dissecting microscope.

Virus-induced foci were counted in nontreated and particulate treated wells. The mean number of focus forming units and standard deviation was determined for each treatment time and significance determined by Student's t-test.

Alterations in Cellular Membrane Composition

Surface Labeling--

For labeling in the presence of phosphate buffered saline (PBS), and the incubation volume adjusted to five ml with the same buffer (7,8). For labeling in the presence of media alone, minimal essential medium (MEM) was employed for washing and final incubation volume adjustment. For labeling with media containing 10 percent FBS, the incubation volume was adjusted to five ml. Galactose oxidase [Sigma Biochemical Type III (125 units/mg) or Grand Island Biological Co. (100 units/mg)] (200-250 µg) was added to give a final concentration of 25 units per dish, and the plates were incubated in a Precision Scientific Co. p^5 Model 2 oven under CO_2-O_2 atmosphere for three hours at 35°C. After incubation, the dish was washed twice with either PBS or MEM medium and the excess incubation mixture was aspirated. One mCi of NaB^3H_4, with S.A. 9 Ci/mmol (New England Nuclear; stored in 0.01 N NaOH solution at -40°C) was added and allowed to stand with occasional shaking for 30 minutes at 37°C. Finally, the cells were detached from the plates with a scraper having a wide rubber blade. The reaction mixtures obtained from the various plates were washed five times with PBS pH 7.4, and centrifuged at 1500 rpm for 10 minutes each time. The pelleted cells were suspended in 200 µl PBS, and aliquots were taken for the glycolipids and glycoproteins determinations. For glycoproteins the cells (10^6) were digested in PBS (100 µl) containing one percent sodium

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dodecyl sulfate (SDS) and five percent β-mercaptoethanol and heated in a water bath at 85°C for 10 minutes.

SDS-Polyacrylamide Gel Electrophoresis--

Electrophoresis was performed according to the method of Weber and Osborn (9). β-galactosidase, urease, albumin, peroxidase and lysosyme with molecular masses of 130,000, 83,000, 68,000, 44,050 and 13,930 respectively were used. The gels were sliced and each slice radioassayed.

Extraction of Syrian Hamster Cells Glycolipids (10)--

An aliquot (100 µl) of surface labeled cells (10^6) was homogenized with 1 ml of methanol for five minutes at room temperature, and chloroform (2 ml) was added so that the final ratio was 30 volumes of chloroform; methanol (2:1, v/v) to 1 volume of cells. The homogenate was left overnight at room temperature for efficient extraction, and then centrifuged at 1500 rpm for ten minutes and the precipitate washed twice with chloroform:methanol (1:1, v/v).

Isolation of Glycolipids (11)--

Following the extraction of lipid from the cells, polar glycolipids (gangliosides) were separated from neutral glycolipids by the procedure of Folch (12). After Folch partition the total upper layer was reduced in volume to one to two ml and dialyzed at 4°C against distilled water for 24 hours. The dialyzed upper phase was evaporated to dryness under N_2, and the residue extracted with a small volume of chloroform:methanol (2:1, v/v). This fraction represents a major part of the gangliosides and contains some neutral glycolipids in minor quantities. The analysis of gangliosides was performed by thin-layer chromatography (TLC) (Kontes/Quantus precoated TLC plates) and developed in a solvent system (tetrahydrofuran: 0.5 percent aqueous KCl, 7:1, v/v). Standard gangliosides were run separately in the same system at the same time. The standard gangliosides were detected with resorcinol reagent. Zones having the same R_f as the standards were scraped with a razor blade and counted for radioactivity. The lower phase, containing the neutral glycolipids, sulfatides, neutral lipid and phospholipid, was evaporated to dryness and the glycolipid fraction separated according to Laine et al (13). The isolated glycolipid fraction was separated into individual neutral glycolipids by TLC as described previously (14,15).

Determination of Cyclic Nucleotide Levels

Incubation with Particulate Samples and Preparation of Cell-free Extracts--

Particulate samples were suspended in the medium at the desired concentrations. Cells were incubated in the medium containing the particulates for various time periods (16,17,18). At the end of the incubation period, the medium was poured off, and the cells were washed twice with five ml phosphate-saline buffer (pH 7.4). Cells were harvested in two ml of five percent trichloroacetic acid (TCA) with the help of a rubber scraper. The cells in TCA were sonicated for 10 seconds and centrifuged at 4°C at 3000 rpm for 20 minutes. The precipitate fraction was dissolved in 1 N NaOH and saved for measurement of protein, which was determined by the method of Lowry (19). The TCA from the supernatant

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fraction was removed by extracting three times with three volumes of water-saturated diethyl ether. The ether layer was aspirated and any traces of ethyl ether remaining after the last extraction removed by heating the aliquots for 10 minutes at 50°C. The pH of the extracts was adjusted to 6.2 by adding 0.1 volume of 1 M acetate buffer, pH 6.5.

Radioimmunoassay of Cyclic Nucleotides--

Succinylated cyclic nucleotides were coupled to keyhole limpet homocyanin as described by Steiner (20). This antigen was injected into rabbits to produce the antiserum to cyclic nucleotides. The antiserum obtained has very high specificity for cyclic nucleotides. Succinylated cyclic nucleotide-tryosyl methyl ester derivatives are iodinated with ^{125}I-labeled cyclic nucleotide derivative (5000 cpm) followed by 50 µl of 1:2000 dilution of antiserum (cyclic GMP) or a 1:4000 dilution of antiserum (cyclic AMP). The other details of this procedure are identical to those described by Steiner (21). This method permits the measurement of cyclic nucleotides in the femtomole range (22,23).

For each measurement, five plates of cells were harvested individually. The concentrations of cyclic nucleotides were measured in duplicate for each plate. The standard errors of the mean were calculated from 10 values.

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SECTION 4

RESULTS

GENERAL RESULTS

Viral-Directed Cellular Transformation

In previous studies, we showed that all forms of asbestos in the unwashed but autoclaved series, with the exception of amosite, signif-cantly increased the frequency of Snyder-Theilen Feline Sarcoma Virus (STFe SV) transformation on human fibroblast cells in vitro (1). Six unknown substances provided by the USEPA were tested in this system.

Samples 1,2,5, and 6 all significantly inhibited virus transformation when cells were treated 2 hours before infection, whereas substance 3 demonstrated significant enhancement of transformation when cells were treated 2 hours before infection. Substance 4 demonstrated inhibitory properties at all 3 dose levels 2 hours post-infection, although 0.1 µg/ml significantly enhanced transformation when cells were exposed 24 hours before infection. All substances, with the exception of substance 5, affected viral-directed transformation in a dose-dependent manner.

Cyclic Nucleotide Ratio

Previous studies in our laboratories demonstrated that asbestos altered the concentration of cyclic nucleotides in human fibroblasts in vitro. Our present studies with the unknown samples indicated that although these samples did induce some differences in cyclic nucleotide response of human cells, there was no predictable dose-response relationship with any given sample. The response of the unknown samples could be roughly divided into 2 groups. Samples 1 and 2 had relatively minor effects and led to ratios which were essentially unchanged from untreated control cells. Sample 3 caused a slight increase over control values at two of the three concentrations examined. In contrast, samples 4,5 and 6 caused a consistent decrease in the cyclic nucleotide ratio at all concentrations tested with the most striking effect being induced by sample 4. Only sample 3 induced a biphasic effect similar to that reported previously with known samples of asbestos whereas samples 4,5 and 6 induced responses in the cyclic nucleotide ratio similar to that observed for chemical carcinogen treated human cells.

Cell Survival

All samples examined induced cytotoxicity at the highest concentration tested (10 mg/ml); however, at lower concentrations a wide range of

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TABLE 1. COMPARATIVE EFFECTS OF UNKNOWN SAMPLES

	Sample Number						
Test	1	2	3	4	5	6	Control†
Cytotoxicity							
LD_{10}	500ng/ml	10µg/ml	--	>1mg/ml	50µg/ml	1µg/ml	100ng/ml
LD_{50}	10µg/ml	50µg/ml	--	>5mg/ml	90µg/ml	20µg/ml	2µg/ml
LD_{90}	150µg/ml	1mg/ml	--	>5mg/ml	500µg/ml	100µg/ml	75µg/ml
Viral (0.1µg/ml)							
-24	3.37*	3.59*	1.51	2.76*	1.86	3.89*	
- 6	5.85*	2.84*	0.34	1.27	1.20	1.88*	
- 2	0.46	4.98*	5.65*	2.69*	1.18	1.39	
+ 2	7.26*	2.39*	0.38	2.75*	1.03	1.31	
+ 6	2.68*	4.95*	1.65	1.36	1.90	3.10*	
+24	5.54*	2.62*	3.00*	0.66	0.52	4.71*	
(1.0µg/ml)							
-24	0.39	0.89	0.98	0.35	2.80*	4.45*	
- 6	3.28*	3.65*	0.27	0.48	0.01	3.54*	
- 2	4.12*	4.97*	3.48*	1.40	1.41	0.91	
+ 2	8.40*	1.06	0.39	4.04*	2.46*	--	
+ 6	3.99*	1.23	0.85	2.50*	2.45*	--	
+24	2.75*	0.00	5.22*	2.86*	1.76	3.45*	
(10.0µg/ml)							
-24	7.61*	5.61*	0.89	0.65	9.67*	0.22	
- 6	2.01*	7.26*	1.86	1.35	--	3.80*	

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	1	2	3	4	5	6
- 2	3.56*	10.76*	1.93	1.17	--	2.82*
+ 2	11.14*	10.85*	0.57	5.55*	--	0.99
+ 6	4.72*	1.94*	0.83	1.89	5.62*	3.78*
+24	0.97	3.91*	0.00	2.97*	5.19*	0.58
cAMP/cGMP (48 hours)						
.01µg/ml	105	100	130	50	60	85
.1	95	90	110	55	90	90
1.0	100	100	85	80	75	90
Polar Glycolipids						
G_{M1}	196	125	108	117	65.7	356
G_{M2}	50	79	108	264	264	40.4
G_{D1A}	58	82.5	101	155	21	84.7
G_{T1}	121	117	69.8	7.99	23.8	53.9
Neutral Glycolipids						
"Glucocerebrosides"	54	105	114	113	102	74.7
Cerebroside	131	62.9	56.6	82	83.1	141
GL-4	401	301	65.6	28.4	119	98
Glycoproteins	357	276	10.8	4.38	1.6	28.2

† Control represents treatment with Canadian chrysotile.
*Significant values for virus studies.

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cytotoxicities were exhibited between samples. All experiments were carried out in duplicate with a total of forty plates seeded per dose of sample used. The total number of each size colony formed as well as total number of colonies per dose was determined by use of an Artek Size Discriminator. Canadian chrysotile mixed was used as a positive control for each study. While none of the unknown samples exhibited the same degree of cytotoxicity as did the Canadian chrysotile; samples 1 and 6 proved most cytotoxic with sample 2 and 5 being less cytotoxic than 1 and 6. Sample number 4 was cytotoxic only at the very highest concentration used (10 µg/ml) and even slightly stimulatory at lower concentrations. The determination of colony size, shape, and density revealed that no difference existed between these parameters and the overall reduction in survival as a function of dose. All samples exhibited a dose-response curve.

With previous studies, we have shown that all asbestos forms alter the cell membrane surface. This effect was particularly marked at 48 and 72 hours. Currently, we have tested six unknown fiber preparations for effects over a 48 hour period on Syrian hamster embryo cells in culture with the following results. With incubator samples 1 and 2 increased the more complex glycolipid accumulation on the cell surface whereas incubation with samples 3-6 resulted in moderate to substantial decreases in the complex ganglioside GT_1 and with unknown 5 and 6 treatments moderate to extensive decreases in GD_{1A}. The neutral glycolipid GL-4, a precursor and potential hydrolysis product of gangliosides, was substantially increased in cells treated with unknowns 1 and 2. Decreases in the surface distribution of this glycolipid was observed with samples 3 and 4 and little or no change in this ganglioside precursor-product was observed in treatments with samples 5 and 6.

A high molecular mass glycoprotein was found in high concentrations on the cell surfaces after treatment with samples 1 and 2. Marked decreases in this glycoprotein fraction was observed with samples 4, 5, and 6 treatments of Syrian hamster embryo cells. Sample 3 was not tested.

CONCENTRATION AND TIME EFFECTS

Cytotoxicity

The dose-response curve for each unknown and for Canadian chrysotile was conducted over a range of 10^{-8} to 10^{-2} grams at log intervals. At the highest concentration used, all compounds proved toxic; however, at the LD_{50} level, a difference of three orders of magnitude was noted between samples. The effect of each sample on both the total number of colonies formed as well as size distribution of the colonies as a function of each dose was determined by use of an Artek Size Discriminator. Figure 1 illustrates the comparative dose-response curve obtained for each unknown tested while Figure 2 illustrates the similarity in response, relative to size, of the colonies formed for our known sample of Canadian chrysotile. Our studies have shown that while all samples tested induced

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to one degree or another differing shapes and sizes of colonies relative to the control, each class of colony formed produced a similar dose-survival curve. Relative to potency, due to the large number of plates per point (40 plates minimal), and number of replicate countings per plate (each plate counted at least 4 times via an automatic colony counter and size discriminator) it is our estimation that under the conditions of these experiments and for this particular normal human fibroblast culture, Canadian chrysotile (control) is more potent than 1 >6 >2 >5 >4 based upon their respective LD_{10}'s, LD_{50}'s and LD_{90}'s. In Appendix 1, figures representing each dose-survival curve relative to control and based upon size of colony formed are to be found.

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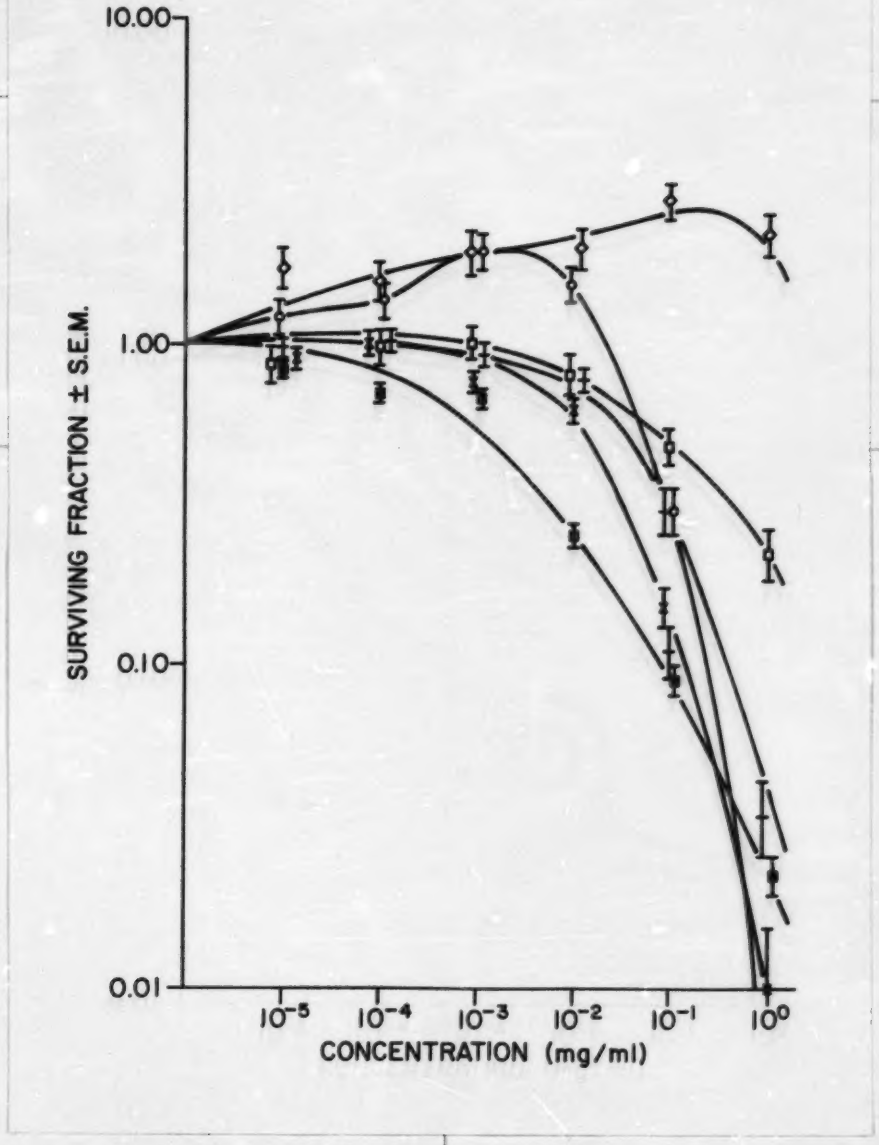

FIGURE 1. The effect of Canadian chrysotile (■) in relation to unknown #1 (x), unknown #2 (□), unknown #4 (◊), unknown #5 (○), and unknown #6 (+) are presented.

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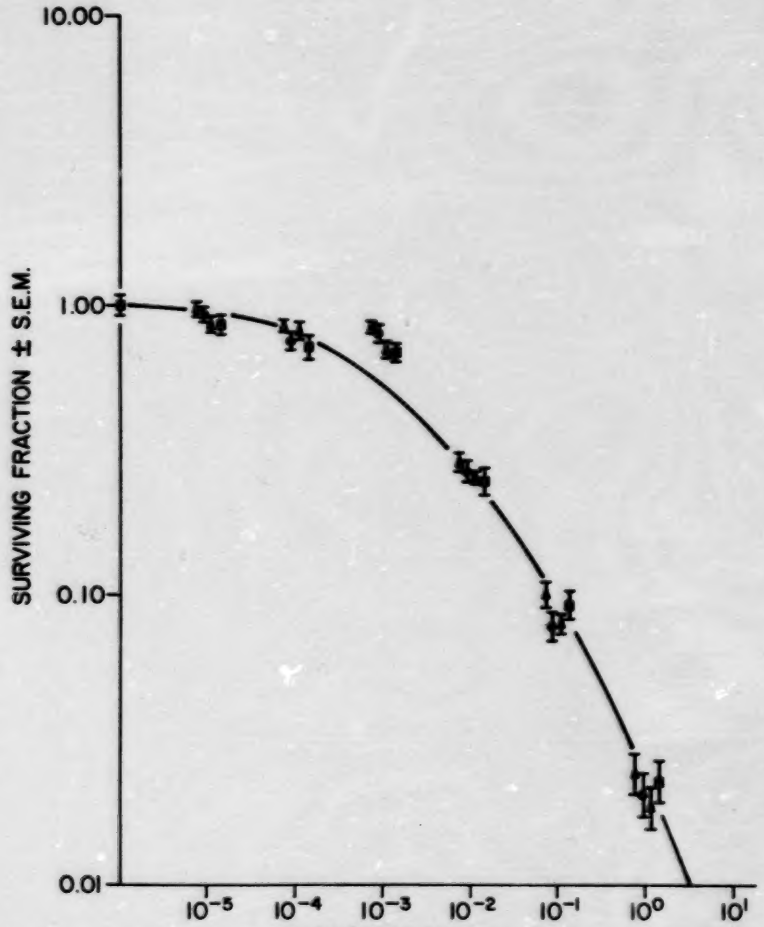

FIGURE 2. These data imply there was no significant difference associated with the morphological size of the colonies counted using the Artek size discriminator at >0.2 mm (Δ), >0.6 mm (●), >1.0 mm (■), and >2.0 mm (▲); after an exposure to Canadian chrysotile.

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Enhancement of Virally Directed Cellular Transformation

Dose-dependent effects on viral-directed cellular transformation were observed for 5 of the 6 substances tested (Figures 3-8).

In those instances where the substance increased the frequency of virus transformation, the greatest increase was of the order 0.1 >1.0 >10 µg/ml. Conversely, when the substance inhibited virus transformation the order was reversed at 10 >1.0 >0.1. It should be pointed out that none of these doses were toxic in that results of cloning efficiencies of treated cells were the same as untreated cells (data not shown).

The inhibition of virus transformation by the unknown substances varied as to the time of application. For example, a) the inhibition effect of substance 1 ranged from 6 hours before infection to 24 hours postinfection; b) #2 from 24 hours before infection to 24 hours post-infection; c) #4 from 24 hours preinfection (10 µg/ml) to 24 hours postinfection while all 3 dose levels were inhibitory at 2 hours p.i.; d) with #5 at 10 µg/ml significant inhibition resulted at all time periods with almost complete inhibition of virus transformation occurring with cells treated 6 or 2 hours preinfection while 1.0 µg/ml inhibited 2 and 6 hours postinfection; e) #6 (at 1.0 and 10 µg/ml) were inhibitory at -6 hours and -2 hours (1.0 µg/ml).

Substances 3,4 and 5 enhanced virus transformation when cells were treated 2 hours preinfection, while substances 1,2 and 6 enhanced transformation postinfection, although substances 1,2,4 and 6 all demonstrated enhancing properties at 24 hours preinfection.

Modification of Cyclic Nucleotide Ratios

The experimental protocol for these studies was essentially identical to that of our previous studies, except that we chose a single exposure time, 48 hours, for all of the samples at three different concentrations (.01, 0.1 and 1.0 µg particulate/ml of medium). This protocol was used because our previous results (Tejwani, et al., J. Env. Toxicol and Pathol. accepted for publication) indicated that there was predictable changes in the ratio of cyclic AMP to GMP at this time after exposure to asbestos.

Our results indicated that although there were some differences in the cyclic nucleotide response of the cells with increasing asbestos concentrations, there was no predictable dose-response relationship with any given sample. However, there were striking differences in the tissue response to different samples. The samples could be roughly divided into 2 groups. Samples 1 and 2 had relatively minor effects, and led to ratios which were essentially unchanged from untreated control cells (Figure 9). Sample 3 caused a slight increase over control values at two of three concentrations. In contrast, samples 4,5, and 6 caused a consistent decrease in the cyclic nucleotide ratio at all concentrations tested. The most striking effects were caused by sample 4 (Figure 10).

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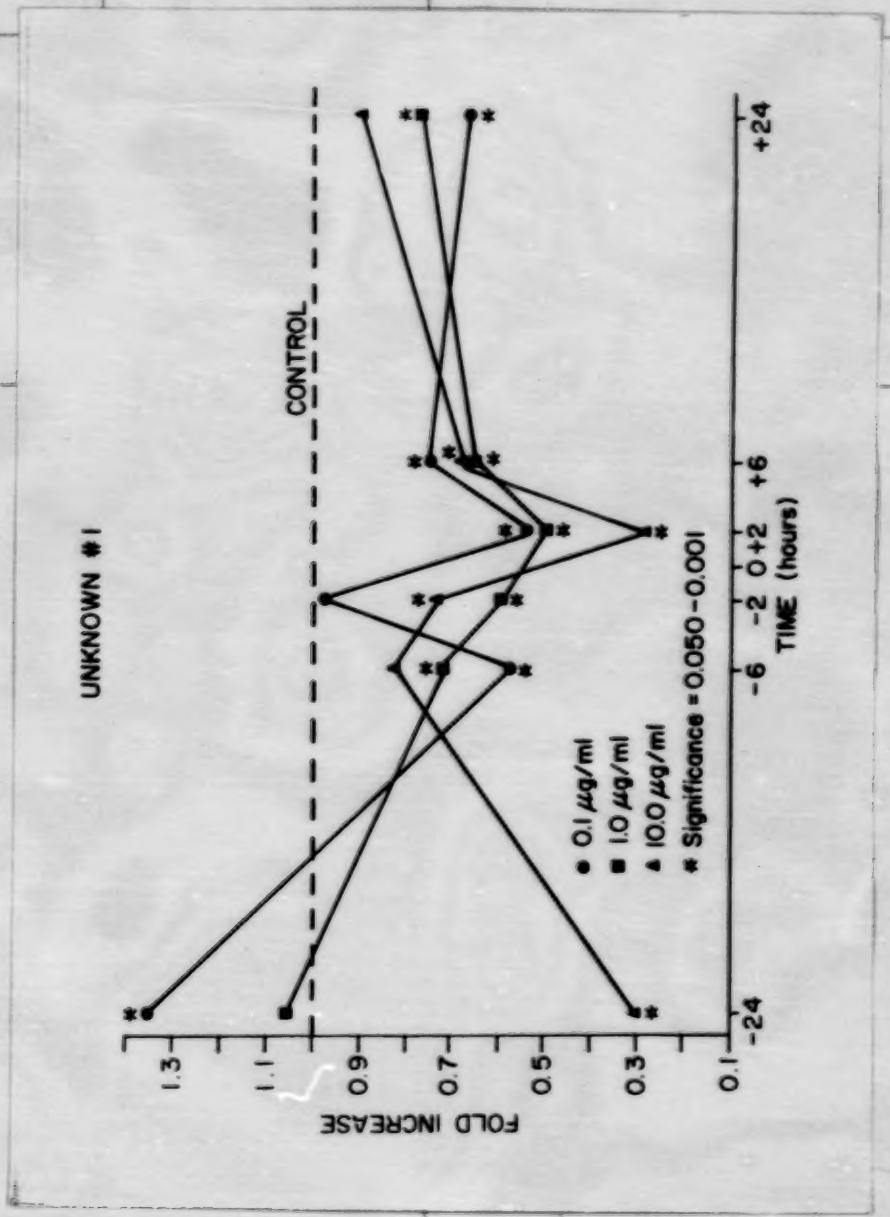

FIGURE 3 Effect of unknown number 1 on STFe SV transformation on human fibroblast cell cultures at 3 concentrations

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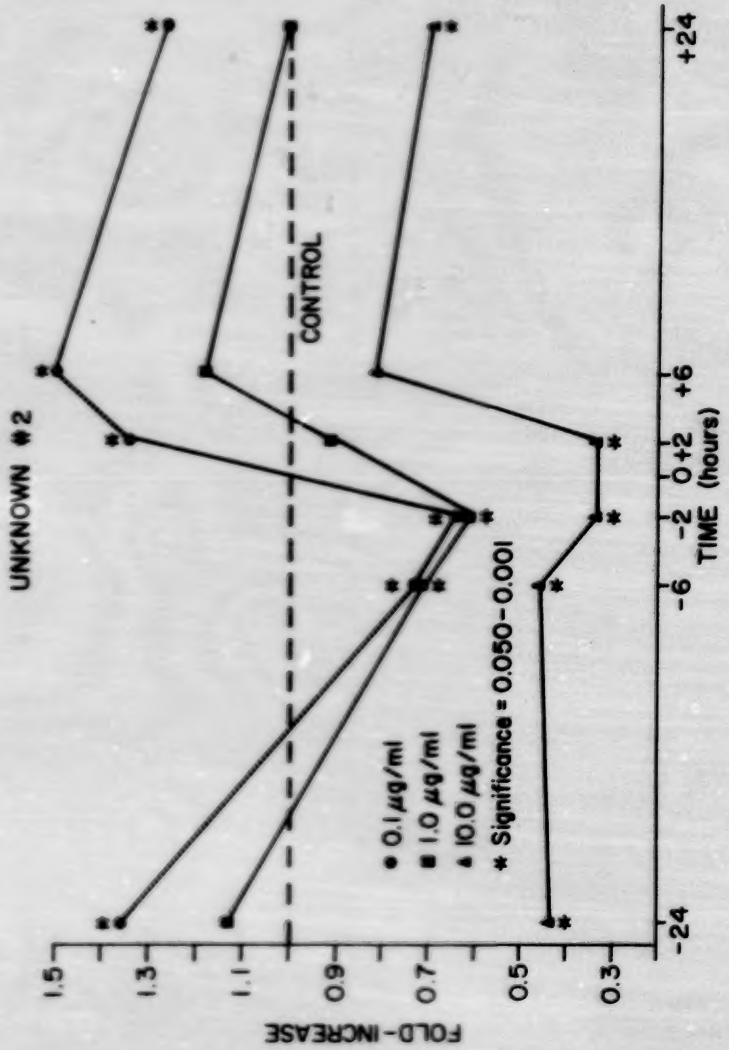

FIGURE 4 Effect of unknown number 2 on STFe SV transformation on human fibroblast cell culture at 3 concentrations

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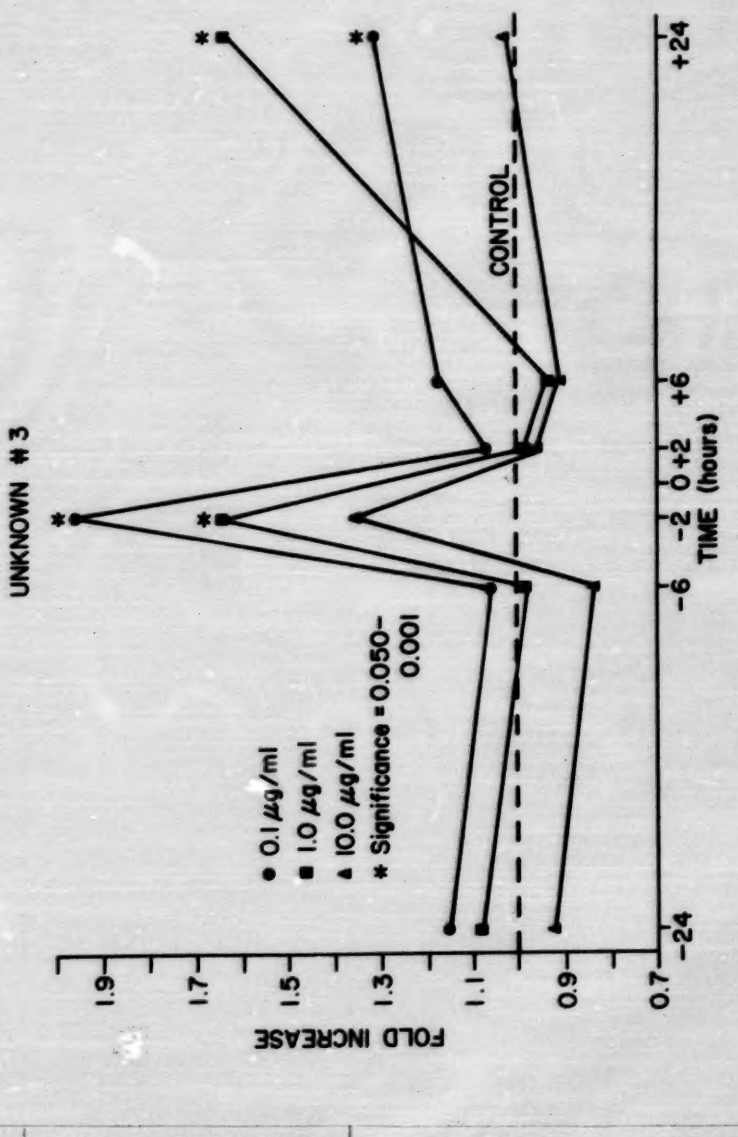

FIGURE 5 **Effect of unknown number 3 on STFe SV transformation on human fibroblast cell culture at 3 concentrations**

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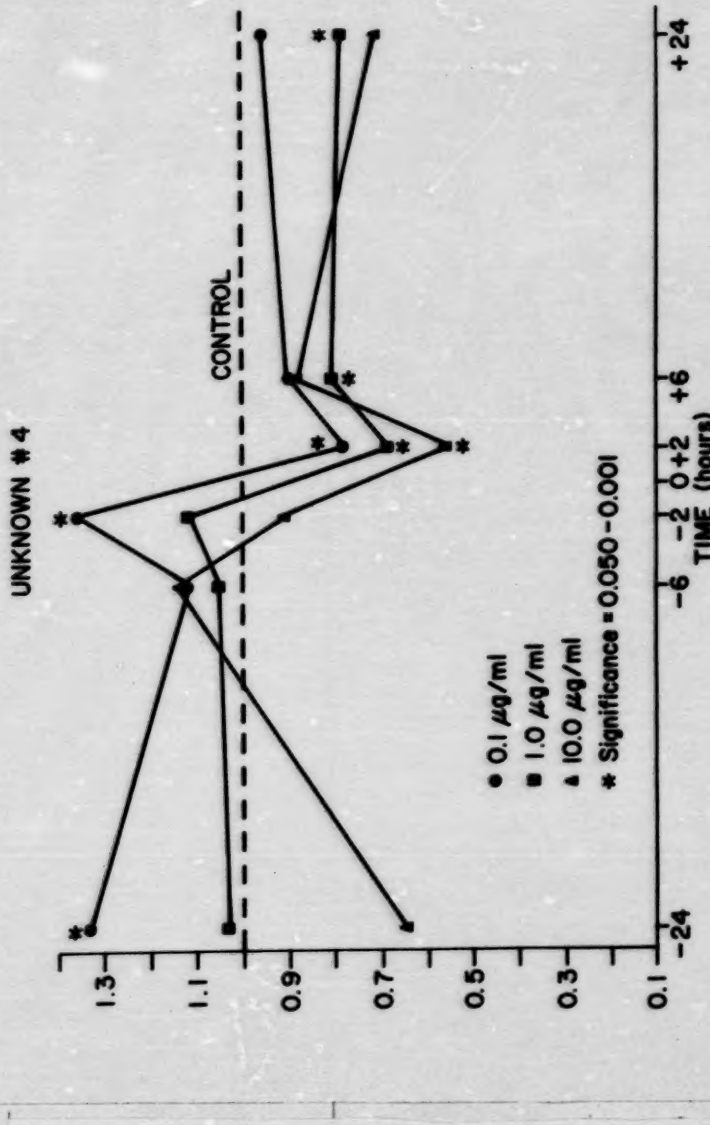

FIGURE 6 **Effect of unknown number 4 on STFe SV transformation on human fibroblast cell culture at 3 concentrations**

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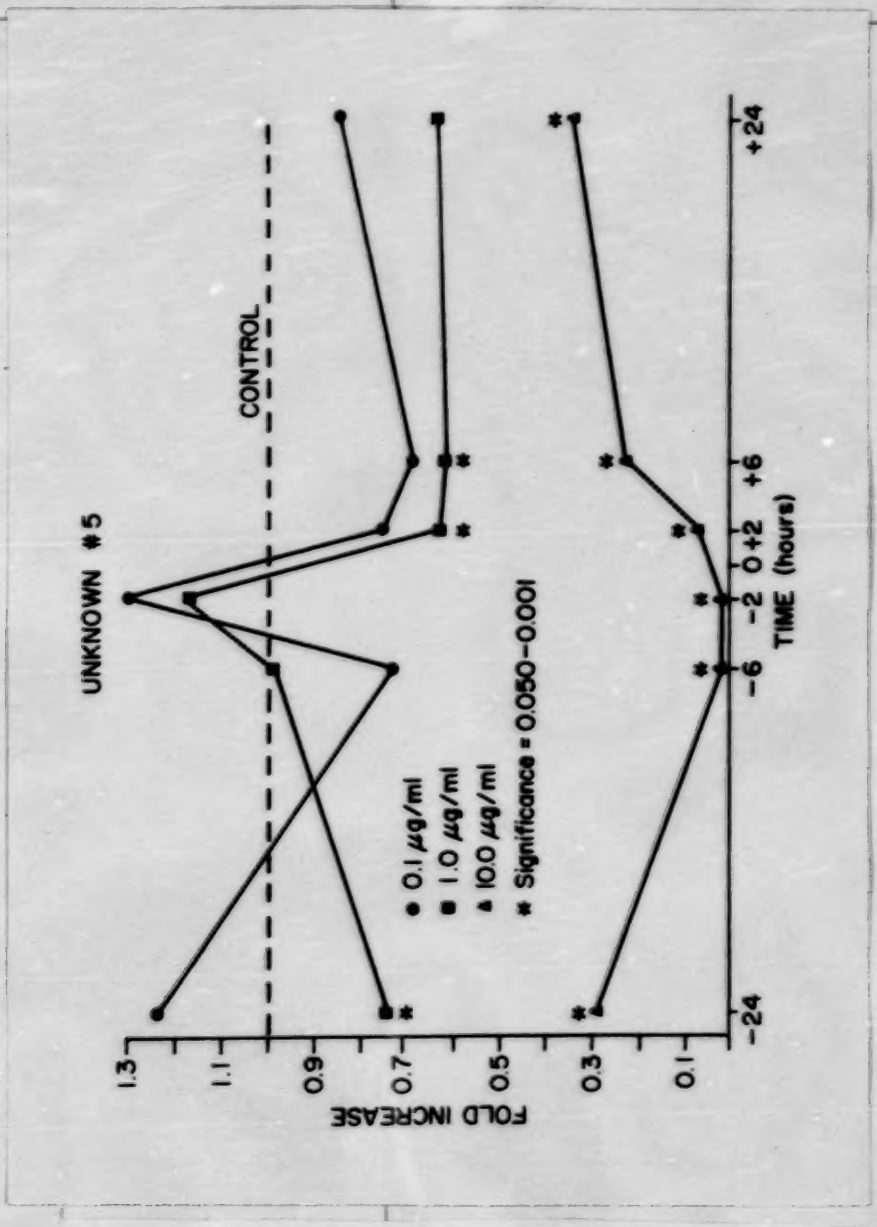

FIGURE 7 Effect of unknown number 5 on STFe SV transformation on human fibroblast cell culture at 3 concentrations

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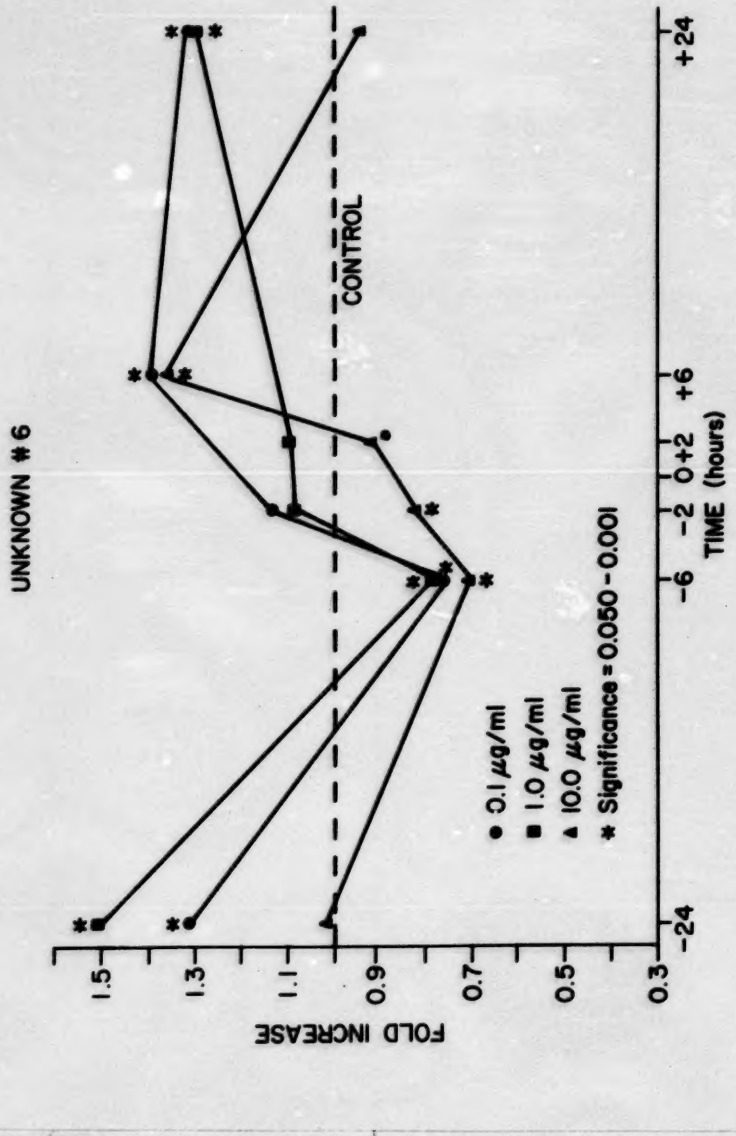

FIGURE 8 Effect of unknown number 6 on STFe SV transformation on human fibroblast cell culture at 3 concentrations

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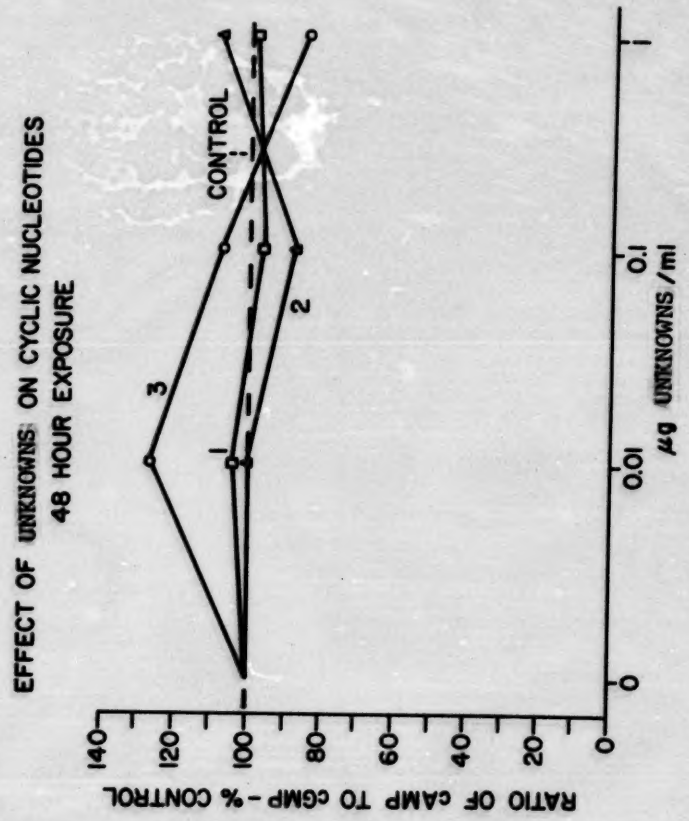

FIGURE 9 **Effect of unknown samples number 1,2 and 3 on the ratio of cyclic AMP to cyclic GMP in normal human fibroblasts.**

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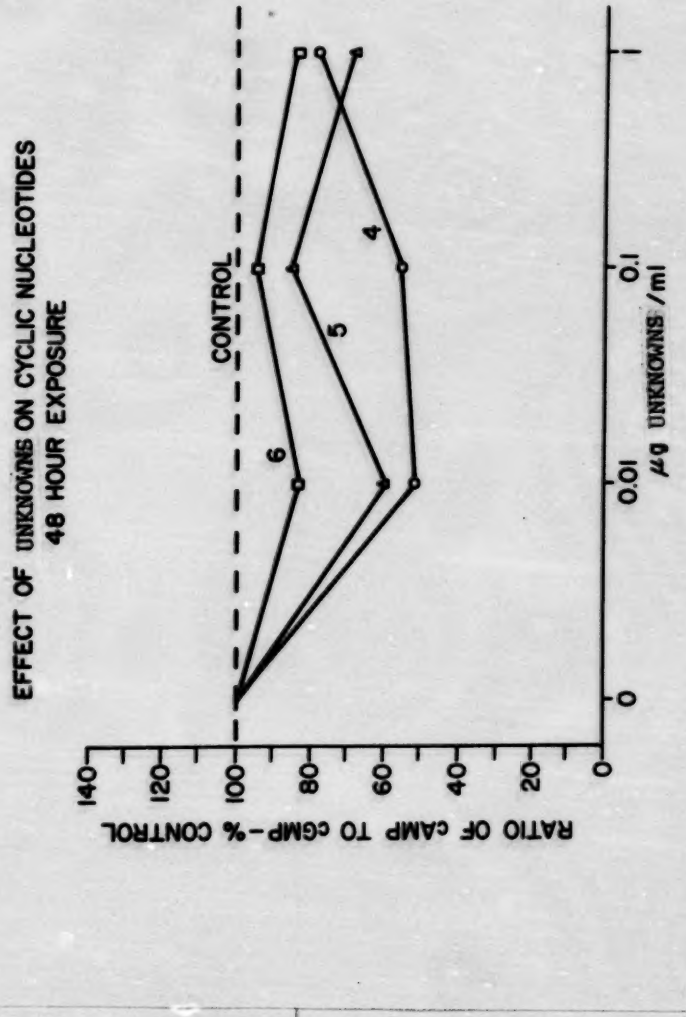

FIGURE 10 Effect of unknown samples number 4,5 and 6 on the ratio of cyclic AMP to cyclic GMP in normal human fibroblasts

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TABLE 2

The relative percentage distribution of surface-labeled polar and neutral glycolipids of Syrian hamster cells treated with chrysotile asbestos and unknown fibers 3, 4 and 5

Polar Glycolipids	Control	Chrysotile (mixed)	Fiber 3	Fiber 4	Fiber 5	Control	Chrysotile	Fiber 6
G_{M1}	23.6 ± 2.47*	13.4 ± 7.14	25.4 ± 9.57	27.7 ± 8.15	15.5 ± 3.74	14.4 ± 11.2	21.1 ± 9.87	51.4 ± 53.5
G_{M2}	32.0 ± 5.6	43.2 ± 29.1	34.4 ± 12.9	34.4 ± 12.9	84.6 ± 9.26	66.4 ± 23.7	53.0 ± 22.1	26.8 ± 27.9
G_{D1A}	25.5 ± 1.98	31.2 ± 16.1	25.7 ± 15.1	39.6 ± 4.03	5.35 ± 3.46	13.7 ± 11.7	12.7 ± 4.72	11.6 ± 14.0
G_{T1}	18.9 ± 4.94	12.2 ± 5.87	13.2 ± 1.08	1.51 ± 1.14	4.50 ± 2.12	5.5 ± 0.85	13.2 ± 7.60	10.2 ± 11.9
Neutral Glycolipids								
"Glucocerebrosides"	73.9 ± 7.47	79.8 ± 5.23	84.4 ± 1.51	83.4 ± 2.65	75.0 ± 6.39	32.1 ± 5.1	14.0	24.0
Cerebroside	17.2 ± 7.55	9.92 ± 3.22	9.73 ± 0.850	14.1 ± 1.91	14.3 ± 5.09	22.1 ± 3.2	14.2	31.2
GL-4	8.89 ± 3.67	10.2 ± 2.04	5.84 ± 0.969	2.53 ± 0.814	10.6 ± 10.7	45.7 ± 8.34	71.8	44.8

*Mean ± Std. Dev., n = 3 unless otherwise noted in ( ).

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Our previous experiments indicated that asbestos has a biphasic effect on the cyclic nucleotide ratios of human fibroblasts. The relatively pure asbestos samples which we tested previously all caused a decrease in the ratio of cAMP to cGMP which lasted for about 24 hours. By 48 hours, all of the initial samples caused a rise of up to 65% in the cyclic nucleotide ratios. None of the unidentified samples, with the possible exception of #3, had such an effect. In fact, the striking decrease in ratio which was observed with these samples was very similar to that which was obtained with chemical carcinogens.

Since we are unaware of the source or purity of the unidentified samples, we can only attempt to identify the reasons for the discrepancies between these and the previously tested samples. First, if there are chemical contaminants associated with the samples, it is possible that these compounds may be acting as chemical carcinogens, and thus causing a decrease in ratio. Secondly, there may be previously untested types of asbestos present, and these may differ in their effects from the standard asbestos forms which we have examined.

Membrane Composition Effects

We observed the effects of the unknown fibers at 10 µg/ml medium, a dose which proved to be effective with all known asbestos fibers. All cultures were incubated with these unknown fibers for 48 hours, the time interval which gave maximal change in both glycolipid and glycoprotein parameters.

Cultured Syrian hamster embryo cells treated with samples 3,4,5 and 6 for 48 hours gave the following findings. Sample 3 treatment resulted in a significant percentage lowering of G_{T1}, cerebroside and GL-4 over that in the control. Sample 4 treatment resulted in a significant rise in percentage label in G concommitant with a large drop in the percentage of labeled ^3H found in G_{T1}. In neutral glycolipids there was a significant drop in GL-4. Sample 5 treatment resulted in a marked lowering in G_{D1A} and G_{T1} and a lesser lowering in G_{M1}. Concommitantly G_{M2} rose significantly. Although sample 6 appeared to initiate a marked rise in G_{M1} percent of ^3H label, due to the large standard deviations no significance can be attached to this observation.

The glycoproteins in the control for samples 1 and 2 (Figure 15) had high cell surface concentrations of low molecular mass surface proteins and concurrently low concentrations of high molecular mass protein. Samples 1 and 2 (Figures 16 and 17) did not have much difference in pattern from the controls.

The controls for samples 3-5 treatment had very high concentrations of a high molecular band of proteins (Figure 18) but no such concentrations of high molecular weight surface protein were in evidence with sample 3-5 treatment (Figure 19, 20, 21). Sample 6 treatment gave similar results when compared to the controls (Figure 22, 23).

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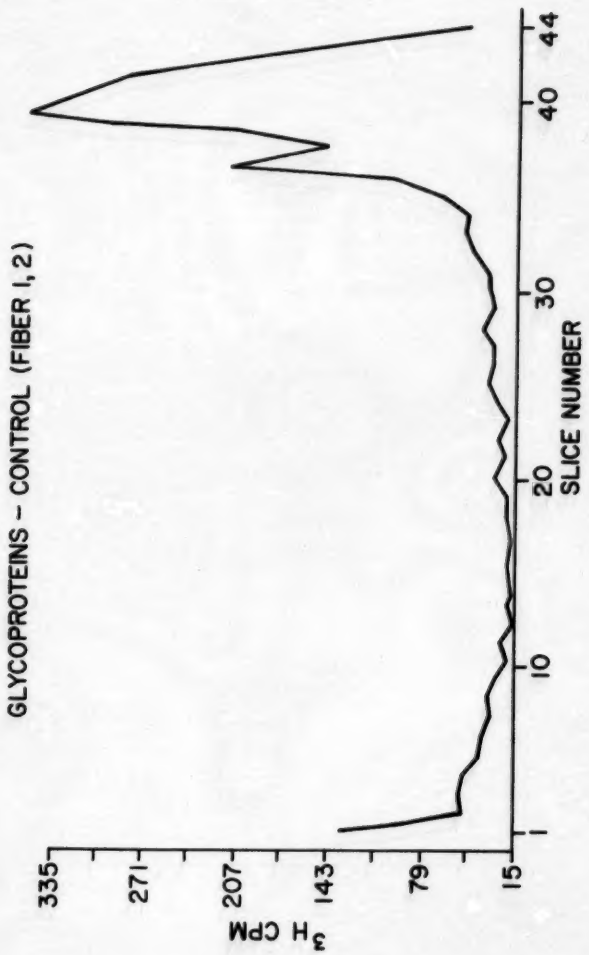

FIGURE 11: Syrian hamster control culture for unknowns 1 and 2: ^{3}H-distribution in electrophoretically separated glycoproteins

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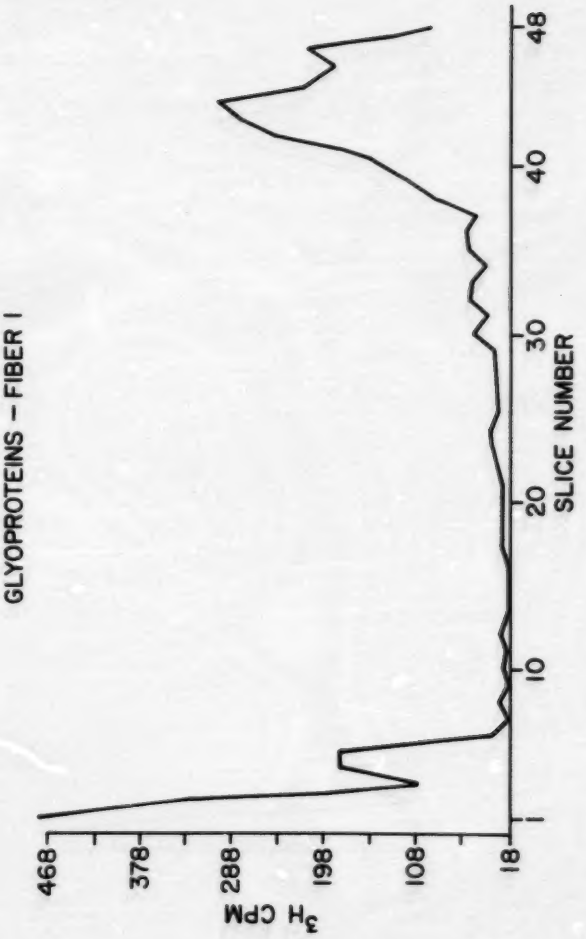

FIGURE 12: Effect of unknown number 1 on ^{3}H-distribution in electrophoretically separated glycoproteins

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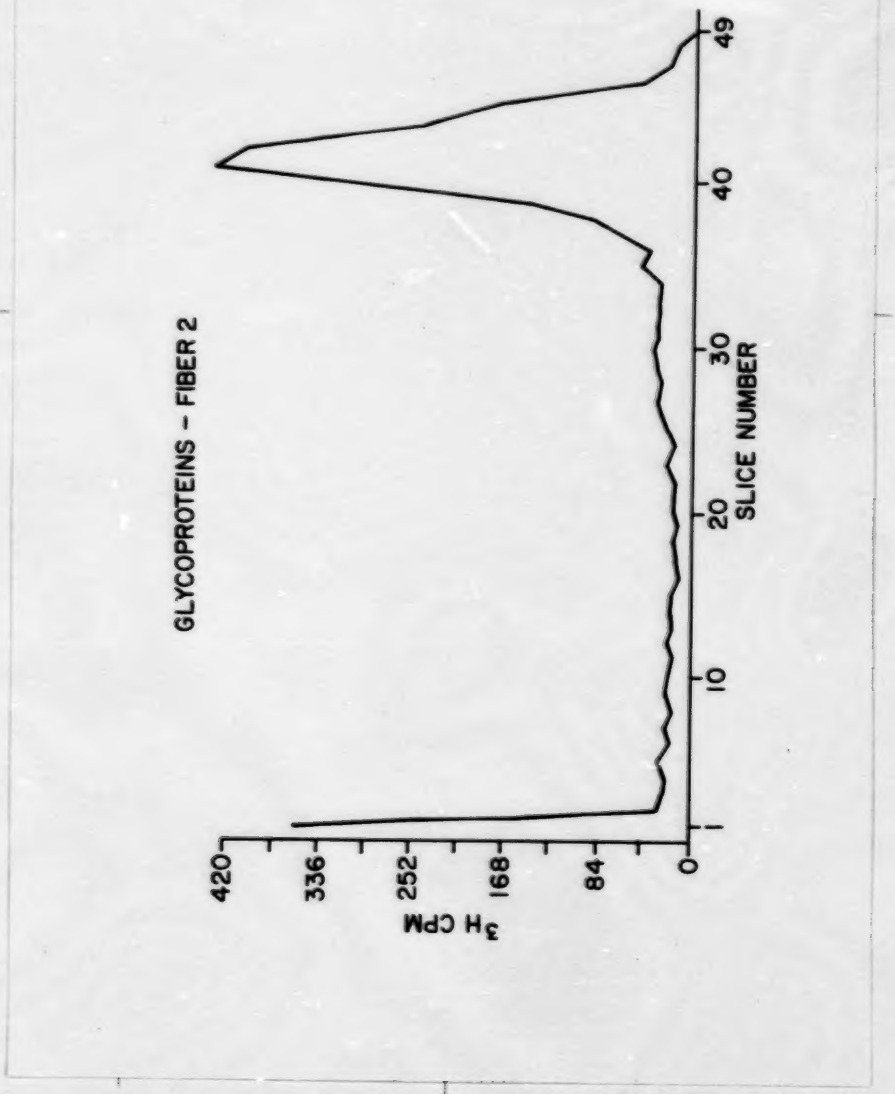

FIGURE 13: Effect of unknown number 2 on ^{3}H-distribution in electrophoretically separated glycoproteins from Syrian hamster embryo cultures

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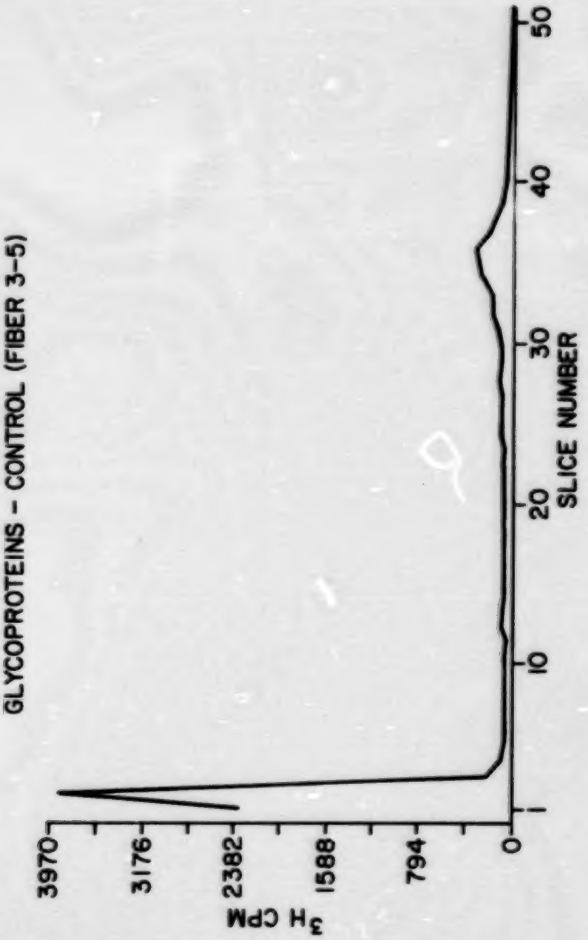

FIGURE 14: Syrian hamster control culture for unknowns 3,4, and 5: ^{3}H-distribution in electrophoretically separated glycoproteins from Syrian hamster embryo cell cultures

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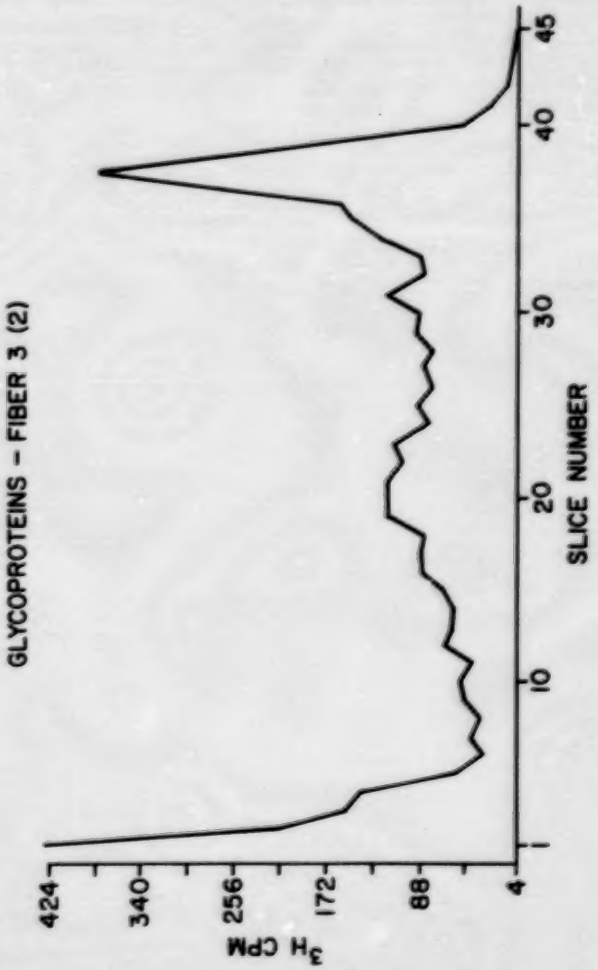

FIGURE 15: Effect of unknown number 3 on ^3H-distribution in electrophoretically separated glycoproteins from Syrian hamster embryo cultures

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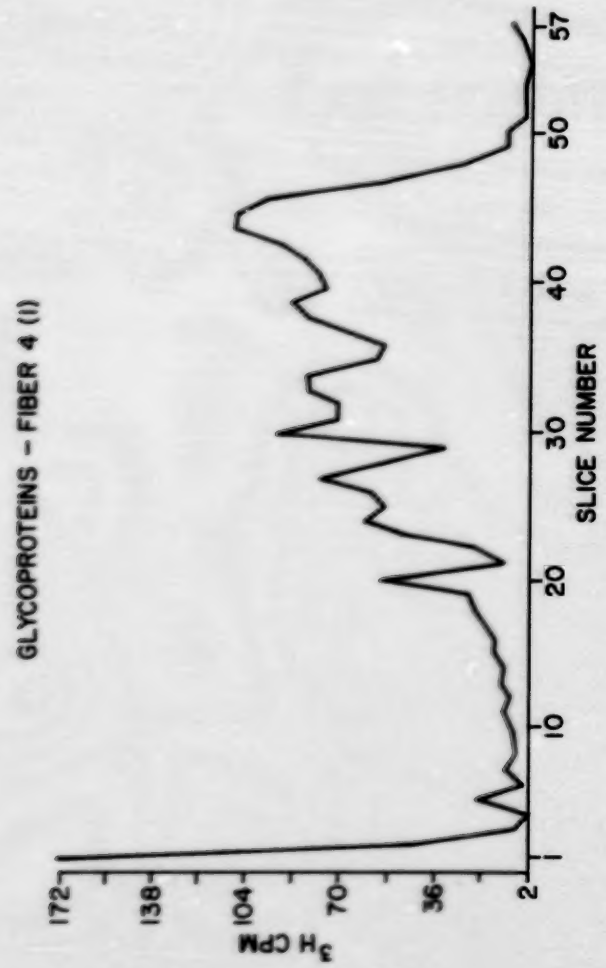

FIGURE 16: Effect of unknown number 4 on ^{3}H-distribution in electrophoretically separated glycoproteins from Syrian hamster embryo cell culture

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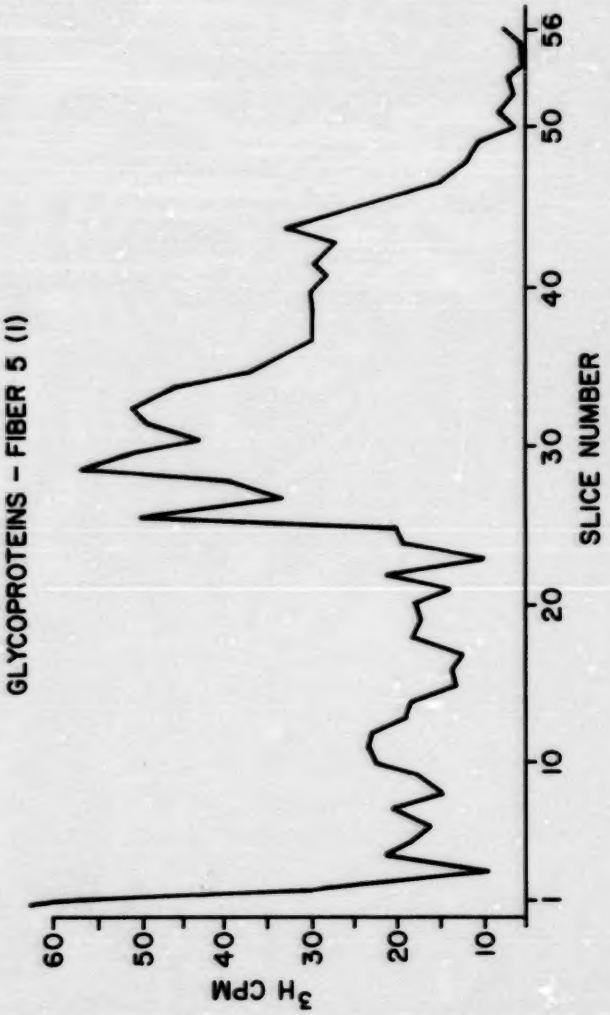

FIGURE 17: Effect of unknown 5 on ^{3}H-distribution in electrophoretically separated glycoproteins from Syrian hamster embryo cell cultures

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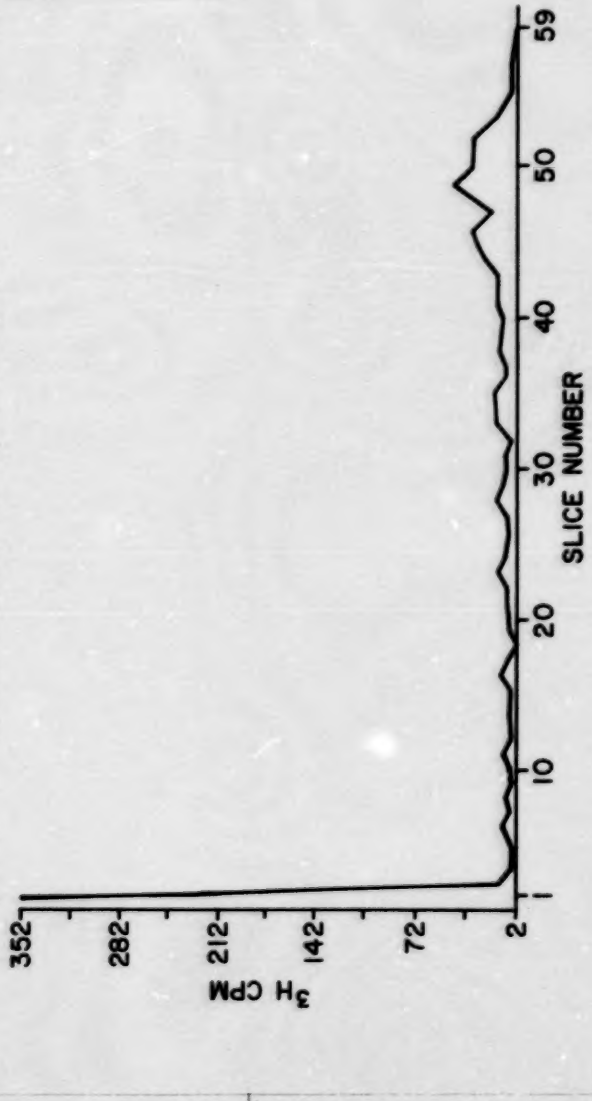

FIGURE 18: Syrian hamster control culture for unknown 6: ^{3}H-distribution in electrophoretically separated glycoproteins

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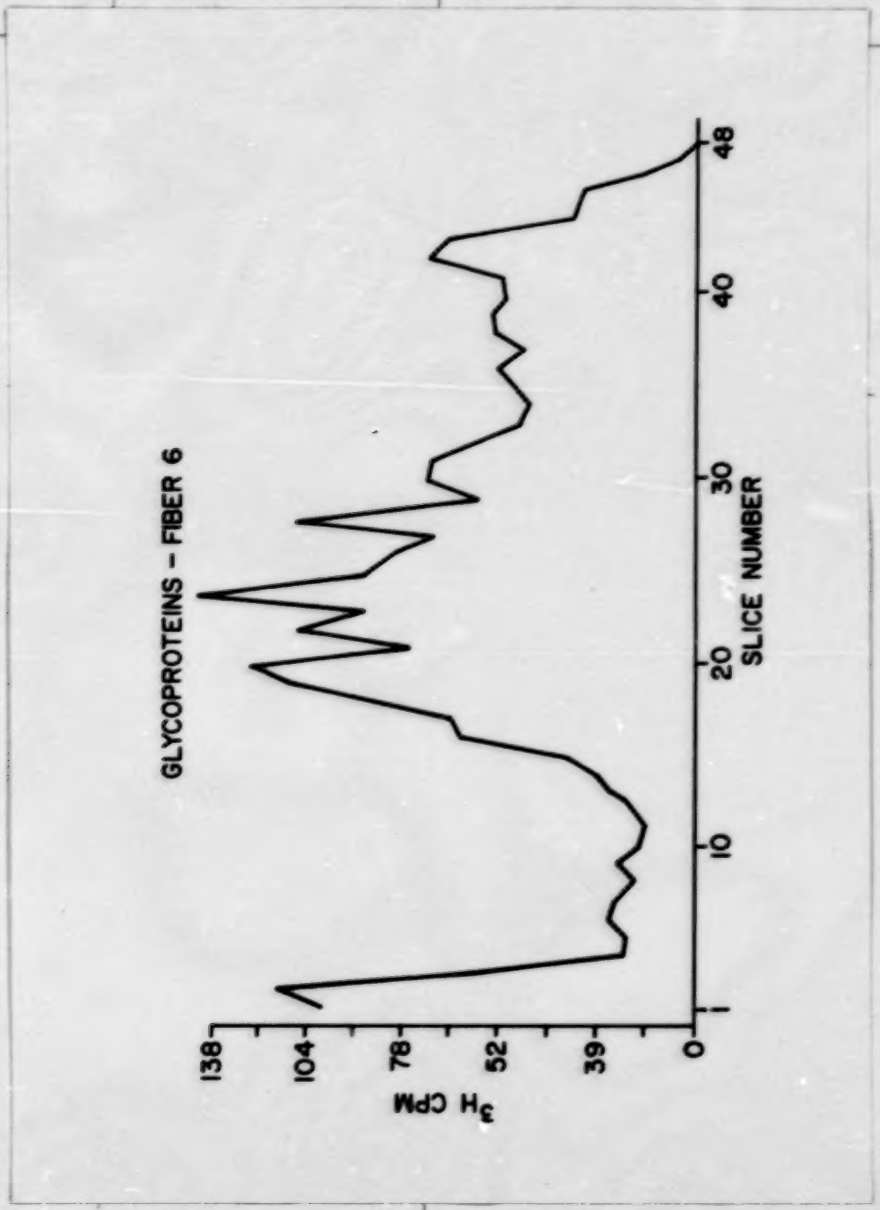

FIGURE 19: Effect of unknown 6 on ^{3}H-distribution in electrophoretically separated glycoproteins from Syrian hamster embryo cultures

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NEGATIVE OR NEGLIGIBLE RESULTS RESPONSE WITH PARTICULATES

It is apparent from these studies that different samples yielded different responses. Unknown sample number 4, while cytotoxic at the highest concentrations tested was relatively noncytotoxic when compared to samples 1,2 and 6 as well as the control (Canadian chrysotile). Relative to viral-directed transformation, all unknowns tested affected the frequency of this parameter with four substances inhibiting and 2 enhancing transformation. With respect to cyclic nucleotide ratio samples, 1,2 and to some degree 3, had little effect on this parameter. The final parameter studied, membrane composition, was modified minimally by interaction with samples 1 and 2.

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APPENDIX

The effect of Canadian chrysotile in relation to the various unknowns are presented for an individual, comparative evaluation. The data for colony size >1.0 mm are presented.

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Table 3: CELL SURVIVAL AFTER TREATMENT WITH CANADIAN CHRYSOTILE

	Control	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}	10^{0}
No. clones per 250 cells	79 ± 3.6	67 ± 3.7	52 ± 2.1	43 ± 1.5	14 ± 0.74	--	--
Absolute plating efficiency	32	27	21	17	5.6	--	--
Relative plating efficiency	100	85	66	54	18	--	--
No. clones per 500 cells	94 ± 2.7	76 ± 1.9	70 ± 2.3	77 ± 2.4	30 ± 1.5	8.5 ± 0.72	2.1 ± 0.31
Absolute plating efficiency	19	15	14	15	6.0	1.7	0.42
Relative plating efficiency	100	81	74	82	32	9.0	2.2
Surviving fraction ± S.E.M.	1.00 ± 0.053	0.83 ± 0.046	0.70 ± 0.037	0.68 ± 0.033	0.25 ± 0.016	0.090 ± 0.0081	0.022 ± 0.0034

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Table 4: CELL SURVIVAL AFTER TREATMENT WITH UNKNOWN #1

	Control	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}	10^{0}
No. clones per 250 cells	17 ± 1.2	13 ± 0.79	15 ± 0.90	12 ± 0.65	9.2 ± 0.45	--	--
Absolute plating efficiency	6.8	5.2	6.0	4.8	3.7	--	--
Relative plating efficiency	100	76	88	70	54	--	--
No. clones per 500 cells	26 ± 1.6	27 ± 1.1	30 ± 1.2	21 ± 1.0	18 ± 1.0	3.9 ± 0.46	0.26 ± 0.12
Absolute plating efficiency	5.2	5.4	6.0	4.2	3.6	0.78	.00052
Relative plating efficiency	100	104	115	81	69	15	1.0
Surviving fraction ± S.E.M.	1.00 ± 0.094	0.90 ± 0.074	1.02 ± 0.083	0.76 ± 0.063	0.62 ± 0.052	0.15 ± 0.020	0.010 ± 0.0046

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Table 5: CELL SURVIVAL AFTER TREATMENT WITH UNKNOWN #2

	Control	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}	10^{0}
No. clones per 250 cells	7.0 ± 1.1	5.7 ± 0.57	5.2 ± 0.58	6.0 ± 0.48	5.2 ± 0.53	--	--
Absolute plating efficiency	2.8	2.3	2.1	2.4	2.1	--	--
Relative plating efficiency	100	81	74	86	74	--	--
No. clones per 500 cells	13 ± 0.78	12 ± 0.80	16 ± 0.67	15 ± 1.0	11 ± 0.69	6.2 ± 0.66	2.9 ± 0.43
Absolute plating efficiency	2.6	2.4	3.2	3.0	2.2	1.2	0.58
Relative plating efficiency	100	92	123	115	85	48	22
Surviving fraction ± S.E.M.	1.00 ± 0.16	0.86 ± 0.12	0.78 ± 0.12	1.00 ± 0.12	0.80 ± 0.11	0.48 ± 0.060	0.22 ± 0.036

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Table 6: CELL SURVIVAL AFTER TREATMENT WITH UNKNOWN #4

	Control	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}	10^{0}
No. clones per 250 cells	5.4 ± 0.75	6.5 ± 0.78	4.8 ± 0.48	7.0 ± 0.67	7.2 ± 0.50	--	--
Absolute plating efficiency	2.2	2.6	1.9	2.8	2.9	--	--
Relative plating efficiency	100	120	89	130	133	--	--
No. clones per 500 cells	6.6 ± 0.75	15 ± 0.89	15 ± 0.80	17 ± 1.0	17 ± 0.80	18 ± 1.3	14 ± 1.4
Absolute plating efficiency	1.3	3.0	3.0	3.4	3.4	3.6	2.8
Relative plating efficiency	100	227	227	258	258	273	212
Surviving fraction ± S.E.M.	1.00 ± 0.18	1.74 ± 0.25	1.58 ± 0.22	1.94 ± 0.28	1.96 ± 0.27	2.73 ± 0.36	2.12 ± 0.32

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Table 7: CELL SURVIVAL AFTER TREATMENT WITH UNKNOWN #5

	Control	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}	10^{0}
No. clones per 250 cells	12 ± 1.9	14 ± 1.0	15 ± 1.1	27 ± 1.1	18 ± 1.2	--	--
Absolute plating efficiency	4.8	5.6	6.0	11	7.2	--	--
Relative plating efficiency	100	117	125	225	150	--	--
No. clones per 500 cells	24 ± 1.7	30 ± 1.8	34 ± 1.6	38 ± 2.1	37 ± 1.4	7.1 ± 1.0	0
Absolute plating efficiency	4.8	6.0	6.8	7.6	7.4	1.4	0
Relative plating efficiency	100	125	142	158	154	30	0
Surviving fraction ± S.E.M.	1.00 ± 0.16	1.21 ± 0.16	1.34 ± 0.17	1.92 ± 0.26	1.52 ± 0.19	0.30 ± 0.047	<0.001

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Table 8: CELL SURVIVAL AFTER TREATMENT WITH UNKNOWN #6

	Control	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}	10^{0}
No. clones per 250 cells	15 ± 1.1	15 ± 1.1	14 ± 0.68	12 ± 0.96	10 ± 0.77	--	--
Absolute plating efficiency	6.0	6.0	5.6	4.8	4.0	--	--
Relative plating efficiency	100	100	93	80	67	--	--
No. clones per 500 cells	32 ± 1.7	31 ± 1.4	36 ± 1.4	33 ± 1.1	27 ± 1.2	3.4 ± 0.53	1.1 ± 0.28
Absolute plating efficiency	6.4	6.2	7.2	6.6	5.4	0.68	0.22
Relative plating efficiency	100	97	112	103	84	11	3.4
Surviving fraction ± S.E.M.	1.00 ± 0.088	0.98 ± 0.084	1.02 ± 0.078	0.92 ± 0.076	0.76 ± 0.064	0.11 ± 0.018	0.034 ± 0.0087

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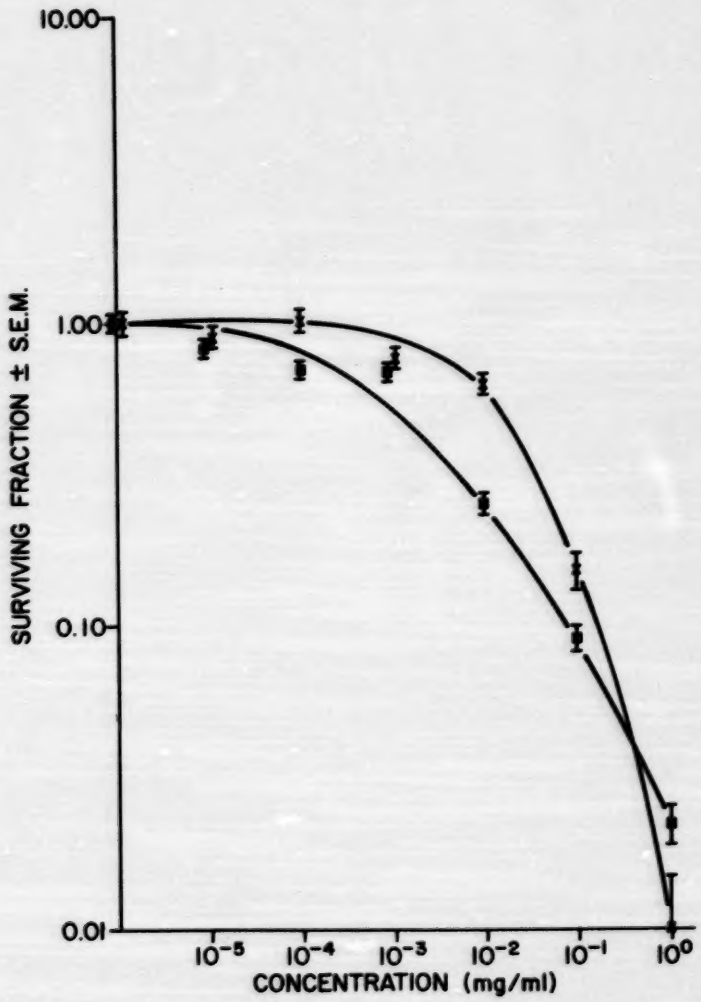

Figure 20: Effect of unknown #1 (x) in relation to Canadian chrysotile (■).

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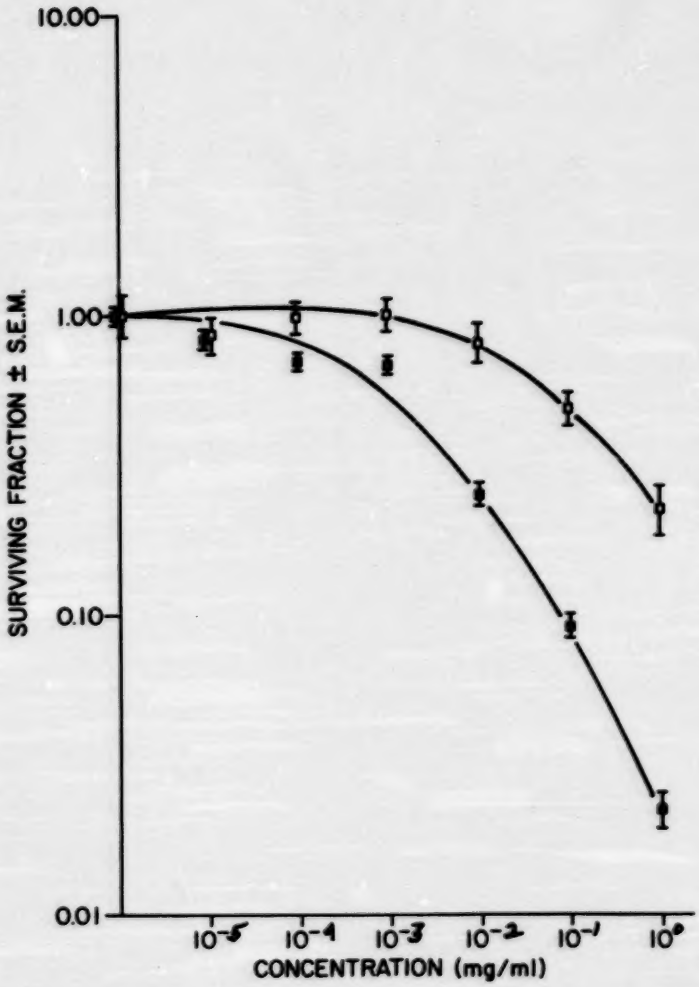

Figure 21: Effect of unknown #2 (□) in relation to Canadian chrysotile (■)

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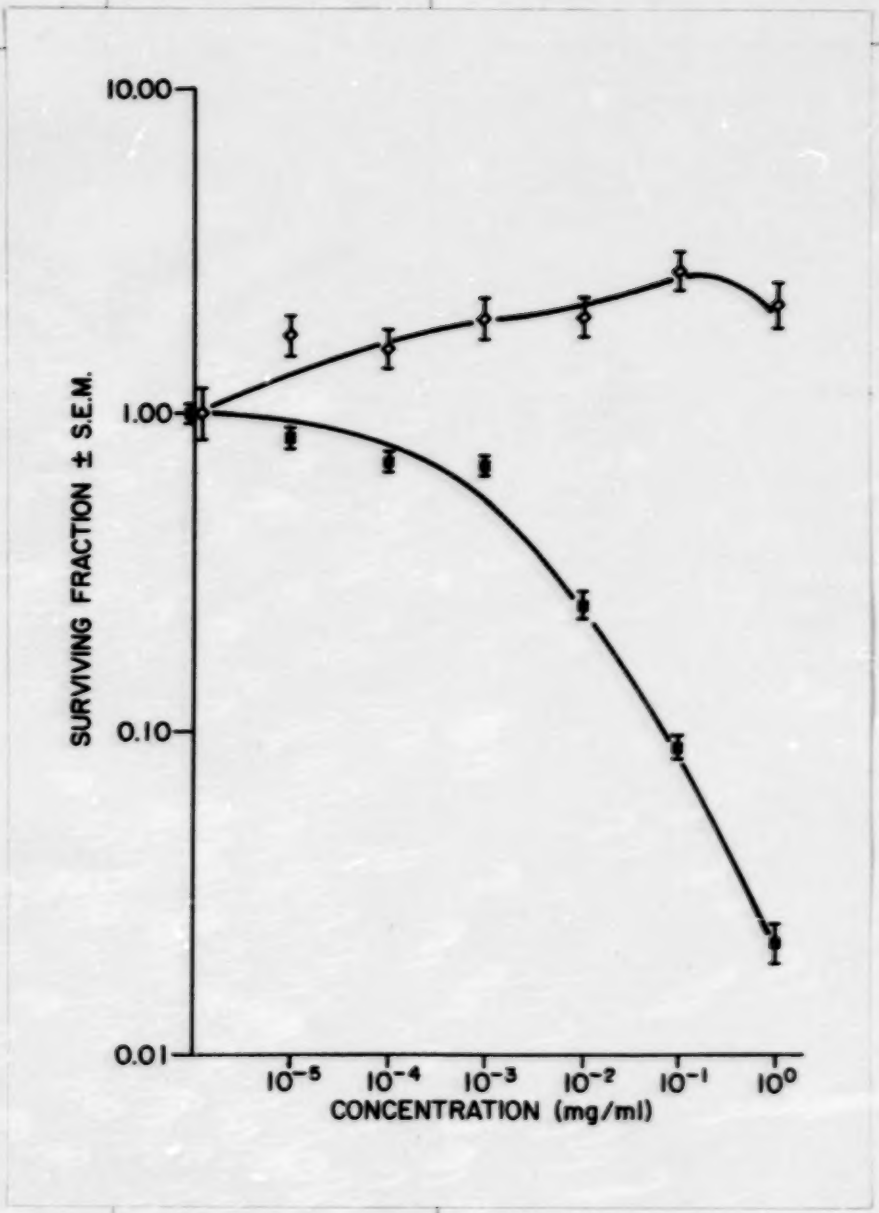

Figure 22: Effect of unknown #4 (◇) in relation to Canadian chrysotile (■)

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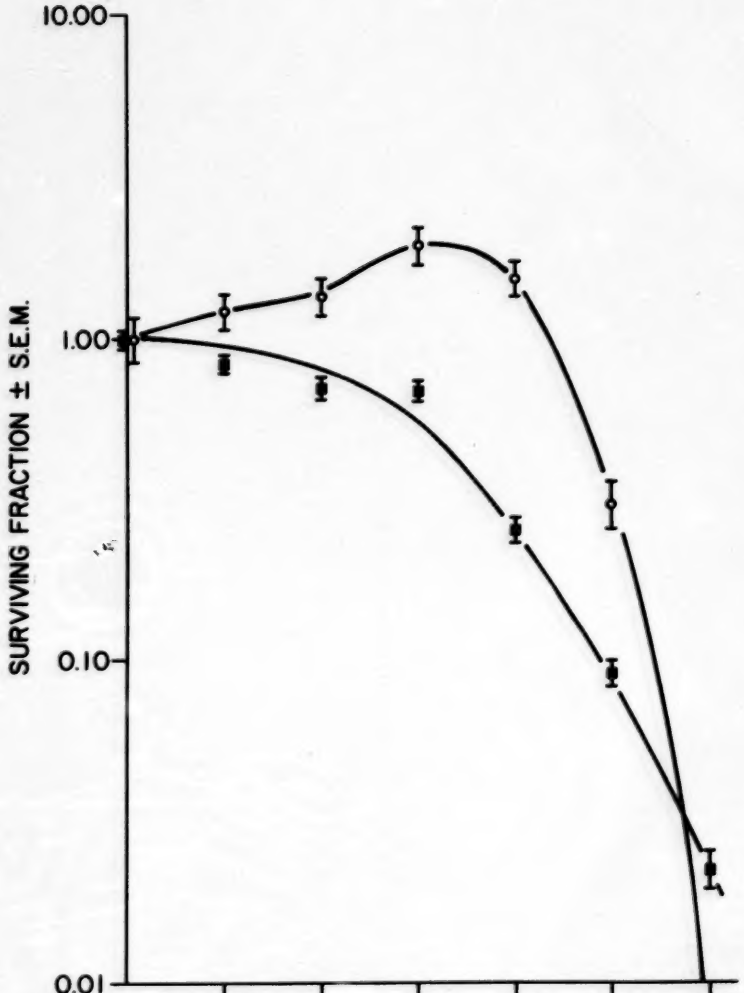

Figure 23: Effect of unknown #5 (o) in relation to Canadian chrysotile (■)

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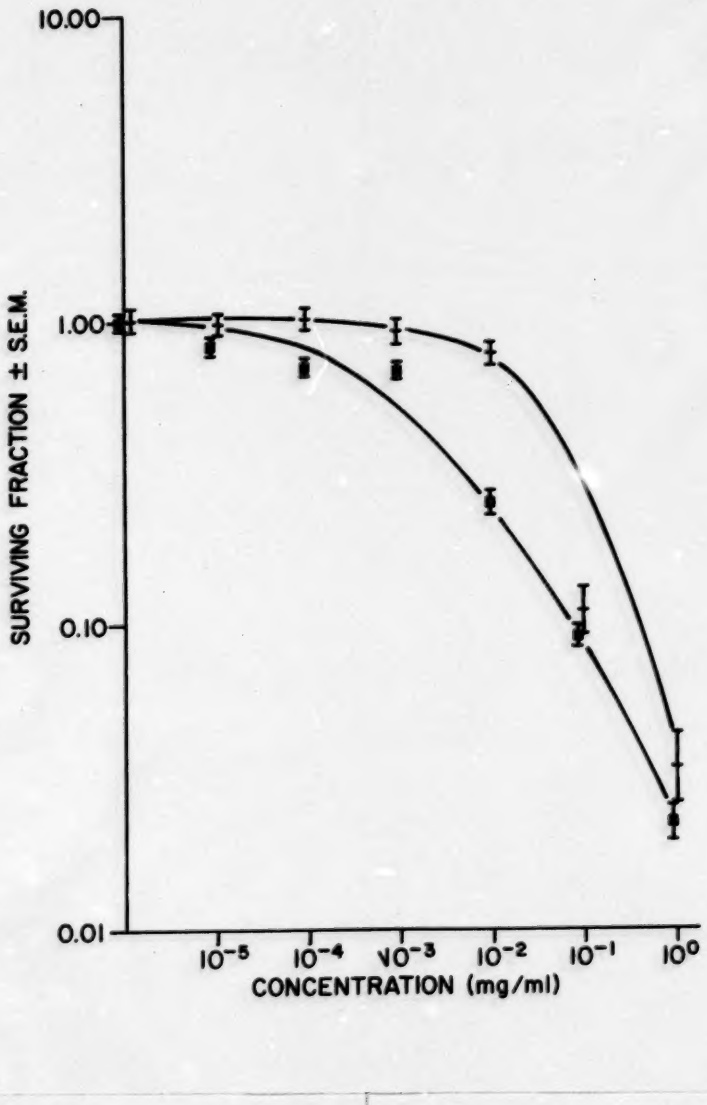

Figure 24: Effect of unknown #6 (+) in relation to Canadian chrysotile (■)

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